

Claim 13 was rejected under 35 U.S.C. § 101 because the invention as claimed allegedly lacks patentable utility, and as disclosed is allegedly inoperative for reasons given in Paper No. 7. Office Action at 2. Applicants respectfully traverse this ground for rejection.

The present invention is directed to a nucleic acid corresponding to ORF-R of HIV-1. The nucleic acid can be used as a diagnostic probe in hybridization assays to detect the presence of HIV-1 in a biological sample. (Specification at page 14, line 11 through page 15, line 8.) The presence of such nucleic acid is, of course, indicative of infection of HIV-1.

The standard for utility is not 100% effectiveness, reliability, or commercial utility of a composition. Rather, it is one of "some" utility. E.I. duPont de Nemours & Co. v. Berkley & Co., 205 U.S.P.Q. 1, 10 n.17 (8th Cir. 1980). Utility under § 101 is clearly shown when a properly claimed invention meets "at least one stated objective." Raytheon Co. v. Roper Corp., 220 U.S.P.Q. 592, 598 (Fed. Cir. 1983), cert. denied, 469 U.S. 835 (1984). An invention need not be perfect in that it need only be useful to some extent, and in certain applications. Carl Zeiss Stiftung v. Renishaw PLC, 20 U.S.P.Q.2d 1094, 1100 (Fed. Cir. 1991). To sustain a rejection for lack of utility,

it must be shown that the claimed invention is "totally incapable of achieving a useful result . . ." Brooktree Corp. v. Advanced Micro Devices, Inc., 24 U.S.P.Q.2d 1401, 1412 (Fed. Cir. 1992) (citations omitted). The Examiner has not met this burden.

The Examiner bears the initial burden of showing *prima facie* that the claims are unpatentable under § 101 because of inoperativeness. Fregeau v. Mossinghoff, 227 U.S.P.Q. 848, 852 (Fed. Cir. 1985). This burden can be met if the asserted utility is not believable on its face to persons skilled in the art in view of the contemporary knowledge in the art at the time the application is filed. When this conclusion is reasonable, the burden is on the applicant to rebut it, if he can, such as by offering evidence. Id. at 852.

In the Office Action, the Examiner repeated the arguments from the previous Office Action, and concluded that

applicant simply locates an open reading frame in the sequence of the clone of the instant application. The specification does not show a utility for any protein supposedly expressed from this open reading frame. Nor does applicant demonstrate a utility for this nucleic acid as a probe.

Office Action at 3. Applicants courteously disagree.

A nucleic acid need not encode a protein to establish the usefulness of the nucleic acid in a hybridization assay.

Usefulness of an invention is shown if any object of an invention is met. Standard Oil Co. v. Montedison S.p.A., 212 U.S.P.Q. 327, 344 (3d Cir. 1981), cert. denied, 456 U.S. 915 (1982). Because applicants' nucleic acid is useful as a nucleic acid probe in hybridization assays to detect the presence of HIV-1 in a biological sample, utility under § 101 is shown.

Nevertheless, ORF-R, also known as ORF-F, *nef*, 3'orf, B, E', or F gene, encodes F protein of HIV-1. See Gallo et al., "HIV/HTLV Gene Nomenclature," Nature, 333, 504 (1988) (Exhibit 1); and Wain-Hobson et al., "Nucleotide Sequence of the AIDS Virus, LAV," Cell, 9-17, 12 (1985) (Exhibit 2). HTLV-III and LAV are strains of the same retrovirus, HIV-1. See Ratner et al., "HTLV-III, LAV, ARV are variants of same AIDS virus," Nature, 313, 636-637 (1985) (Exhibit 3).

In support of the argument that applicants' nucleic acid would not be useful as a probe in hybridization assays to detect HIV-1, the Examiner first stated that

applicant [does not] set forth the conditions under which the nucleic acid would serve as a specific probe having a demonstrable utility.

Office Action at 4.

The specification need not include that which is already known by and available to the public. Paperless Accounting, Inc. v. Bay Area Rapid Transit System, 804 F.2d 659, 664 (Fed. Cir. 1986). In fact, techniques that were old and well-known when the application was filed need not be included in the

specification, and are preferably omitted. Spectra-Physics, Inc. v. Coherent, Inc., 827 F.2d 1524, 1534 (Fed. Cir. 1987).

Because hybridization techniques were well known in the art at the time the application was filed, such techniques need not be described in the specification to show usefulness of the claimed invention.

For example, applicants teach that hybridization assays using nucleic acid probes for Hepatitis B virus were known in the art. (Specification at page 14, lines 29-32.) Hahn et al., cited by the Examiner, describe the use of an HTLV probe in hybridization assays known in the art as of November 8, 1994. Hahn et al. at 168. Arya et al., "Homology of Genome of AIDS-Associated Virus With Genomes Of Human T-Cell Leukemia Viruses," Science, 225, 927-930 (August 31, 1984) (Exhibit 4), also describe hybridization experiments between HTLV-I and -II and HTLV-III.

Moreover, the use of a probe corresponding to the U3 and R regions of the LTR of HIV-1 in a hybridization assay to detect the presence of HIV-1 in a biological sample is discussed in Alizon et al., "Molecular Cloning of Lymphadenopathy-Associated Virus," Nature, 312, 757-760 (1984) (Exhibit 5). This reference was published on December 20/27, 1984, and was submitted for publication September 20, 1984.

pLAV13, a recombinant carrying an insert encoding the R and U3 regions of the LTR of LAV, was used as a probe in a series of filter hybridization experiments. Uninfected cultures and DNA from uninfected lymphocytes or from normal liver proved negative when screened with the probe under the same hybridization conditions. However, Southern blots of LAV-infected T-lymphocytes and CEM cells showed positive results when screened with the probe. Alizon et al. at 758.

Thus, as of September 20, 1984, one of skill in the art was capable of using an HIV-1 probe in hybridization assays to detect the presence of HIV-1 in a biological sample.

Continuing, the Examiner stated that applicants' nucleic acid lacked utility as a hybridization probe because

[a]s the nucleic acid sequence claimed overlaps with the 3'LTR, it has not been demonstrated under what conditions the nucleic acid sequence would serve as a specific probe for HIV. Applicant is provided with a reference by Hahn et al. . . . Figure 4 notes that when HIV is used as a probe, under certain conditions of hybridization, the LTR region of HtLV-1 appears to cross-hybridize.

Office Action at 4.

Applicants describe above hybridization conditions, known in the art at the time the application was filed, that are useful for the claimed invention.

Hahn et al. refer to a hybridization experiment in which nucleic acid fragments of two strains of HTLV-I and one strain of HTLV-II were first separated on Southern blots. The fragments were then hybridized with the full-length HTLV-III clone. Hahn et al. at 168.

Hahn's results showed that a restriction fragment of the first HTLV-I strain containing exclusively pX sequences, and a corresponding fragment of the second HTLV-I strain containing pX and LTR sequences, faintly hybridized with the full-length HTLV-III clone. Hahn et al. at 168. The corresponding region of HTLV-II showed no hybridization.

The "pX" region of HTLV-I and -II is not the same as the ORF-R region of HIV-1 because the ORF-R region (or *nef*) is unique to HIV. The genomes of HTLV-I and -II do not contain a corresponding region. See Gallo et al., chart of HTLV-I, HTLV-II, and HIV genes (Exhibit 1). Therefore, Hahn et al. do not suggest that applicants' nucleic acid, when used as a probe in a hybridization assay, will detect the presence of HTLV-I or -II, rather than HIV-1, in a biological sample.

Furthermore, the ORF-R region of HIV-1 has minimal homology with the corresponding region of HIV-2. Guyader et al., "Genome Organization and Transactivation of the Human Immunodeficiency Virus Type 2," Nature, 326, 662-669, 666 (1987) (Exhibit 6). This minimal homology is primarily due to a large insertion in the amino terminus of HIV-2. Guyader et al. at 666. Consistent with the lack of homology between the nucleotide sequences of

the F proteins of HIV-1 and HIV-2, the F proteins of the two viruses also show a minimal homology of 37.7%. Guyader et al. at 666.

Because applicants' nucleic acid is not found in HTLV-I or HTLV-II, and because the nucleic acid has minimal homology with the corresponding region of HIV-2, one of skill in the art at the time the application was filed would reasonably conclude that the nucleic acid, when used as a probe in a hybridization assay, will detect the presence of HIV-1 in a biological sample.

Applicants' invention satisfies the threshold "some" utility standard set forth by the court in E.I. duPont. Withdrawal of this ground for rejection is respectfully requested.

It is acknowledged that this amendment is submitted after final rejection of the application. Because this amendment is believed to place the application in condition for allowance, applicants respectfully request entry thereof by the Examiner.

Reconsideration and reexamination of this application, and allowance of the pending claim at the Examiner's convenience, are courteously requested.

The Commissioner is hereby authorized to charge any fees associated with this Amendment to our Deposit Account No. 06-0916. If a fee is required for an Extension of Time under 37 C.F.R. § 1.136 not accounted for above, such extension is requested and should also be charged to our Deposit Account.

Respectfully submitted,

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HIV/HTLV gene nomenclature

SIR—The complexities of the genomes of human retroviruses (the human T-cell leukaemia viruses, HTLV-I and HTLV-II, and the AIDS-causing human immunodeficiency viruses, HIV-1 and HIV-2) are being unravelled at a rapid pace which is likely to continue and expand. In addition to containing a large ensemble of positive and negative regulatory genes that orchestrate virus expression, these viruses are also remarkable in that they seem to have converged onto parallel regulatory pathways. Two of the regulatory genes of the immunodeficiency viruses are analogous to the two regulatory genes of the leukaemia viruses, although their detailed mechanisms of action may be quite different. Deciphering the modes of action of the regulatory genes of these viruses is crucial to the understanding of their pathogenesis as well as to development of therapeutic agents. Because of the tremendous activity in this field, more than one name has sometimes been given to a single gene and the same name may also apply to more than one gene. In the interest of the many new investigators entering the field for the first time, we feel it is important that we reach a standard nomenclature for all known genes of HIV and HTLV. We propose the scheme outlined in the table.

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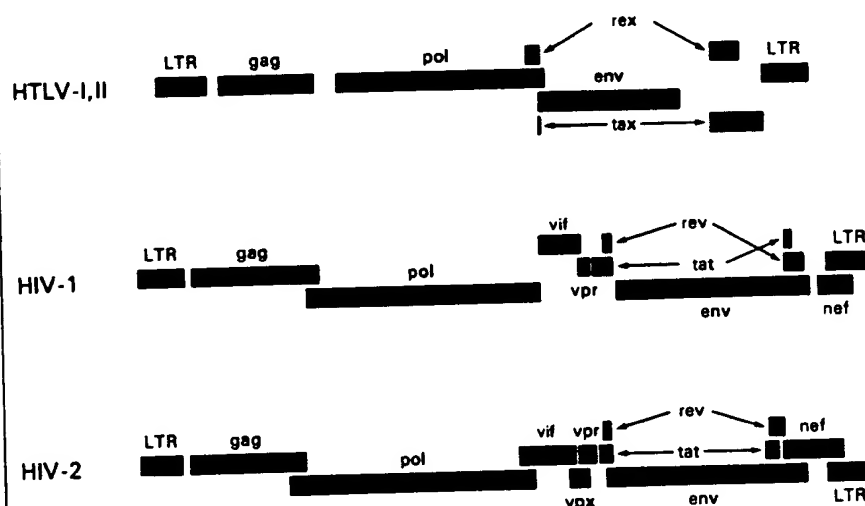
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Proposed name (and derivation)	Previous names	Molecular mass ($\times 10^{-3}$)	Known function
HTLV-I and HTLV-II genes:			
<i>tax</i> ₁ (transactivator)	<i>x-lor</i> , <i>p40x</i> , <i>tat</i> ₁ , <i>tat</i> ₂ , <i>TA</i>	41, 41, 42 38	Transactivator of all viral proteins
<i>rex</i> ₁ (regulator of expression of <i>rex</i> ₂ virion proteins)	<i>pp27x</i> , <i>tel</i>	27 25	Regulates expression of virion proteins
HIV genes:			
<i>tat</i> (transactivator)	<i>tat-3</i> , <i>TA</i>	14	Transactivator of all viral proteins
<i>rev</i> (regulator of expression of virion proteins)	<i>art</i> , <i>trs</i>	19, 20	Regulates expression of virion proteins
<i>vif</i> (virion infectivity factor)	<i>sor</i> , <i>A</i> , <i>P'</i> , <i>Q</i>	23	Determines virus infectivity
<i>vpr</i> (<i>R</i>)	<i>R</i>	?	Unknown
<i>nef</i> (negative factor)	<i>3'orf</i> , <i>B</i> , <i>E'</i> , <i>F</i>	27	Reduces virus expression, GTP-binding
<i>vpx</i> (<i>X</i>) (only in HIV-2 and SIV)	<i>X</i>	16, 14	Unknown



Vpr and *vpx* are temporary names and may be changed when more information about their functions is available. Subscripts 1 and 2 would be used to distinguish genes of HIV-1 and HIV-2 (for example, *rev*₁ and *rev*₂). It is expected that genes of the simian viruses (STLV-I, SIV) would follow similar nomenclature with the subscripts STLV or SIV as appropriate.

Estimating the incubation period for AIDS patients

SIR—The nonparametric analyses of the data on transfusion-related AIDS considered by Medley *et al.*¹ indicate problems of identifiability. With data obtained by retrospective determination of the time of infection for diagnosed AIDS cases, it is only possible to estimate the early part of the incubation distribution up to a constant of proportionality. The same applies to the total number of infections by blood transfusion before any given time. The transfusion data themselves are unable to discriminate between high infection rates coupled with long incubation times on the one hand, or low infection rates and short incubation times on the other.

As do Medley *et al.*¹, we postulate a function $h(x)$ which specifies the increase over time of the number of HIV-infected individuals who eventually develop AIDS, and a probability density function $f(s)$ for the incubation time of those individuals. The corresponding likelihood function can be maximized jointly with respect to h and f . As the likelihood depends only on the product of h and f , it is not possible to estimate either of these functions completely; they may be individually estimated only up to constants of proportionality c and c^{-1} , respectively. Nonparametric estimates of the proportion of eventual AIDS cases that are diag-

nosed within t years of infection, $F(t) = \int_0^t f(u)du$, are given in the figure for the three age groups considered by Medley *et al.*¹ In this figure we show the estimates of $F(t)$ so that for each group, $c = F(7.5)$. For the children, the levelling of the estimate of $F(t)$ by about 3.5 years suggests that the whole of the distribution of incubation times has been seen; it may then be reasonable to suppose that $c = 1$ but, as also noted by Medley *et al.*¹, a second wave of incubation times that exceed 7.5 years is not excluded by these data. For the other two age groups, there is nothing in the transfusion data themselves to suggest a value for c . As a consequence, it is impossible to place any upper bound on the median incubation time. To estimate this,

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Nucleotide Sequence of the AIDS Virus, LAV

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Summary

The complete 9193-nucleotide sequence of the probable causative agent of AIDS, lymphadenopathy-associated virus (LAV), has been determined. The deduced genetic structure is unique: it shows, in addition to the retroviral gag, pol, and env genes, two novel open reading frames we call Q and F. Remarkably, Q is located between pol and env and F is half-encoded by the U3 element of the LTR. These data place LAV apart from the previously characterized family of human T cell leukemia/lymphoma viruses.

Introduction

The recent onset of severe opportunistic infections among previously healthy male homosexuals has led to the characterization of the acquired immune deficiency syndrome (AIDS) (Gottlieb et al., 1981; Masur et al., 1981). The disease has spread dramatically, and new high-risk groups have been identified: patients receiving blood products, intravenous drug addicts, and individuals originating from Haiti and Central Africa (Piot et al., 1984). AIDS is a fatal disease, and there is at present no specific treatment. The causative agent was suspected to be of viral origin since the epidemiological pattern of AIDS was consistent with a transmissible disease, and cases had been reported after treatment involving ultrafiltered anti-hemophilia preparations (Daly and Scott, 1983). A decisive step in AIDS research was the discovery of a novel human retrovirus called lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983). The properties of the virus consistent with its etiological role in AIDS are: the recovery of many independent isolates from patients with AIDS or related diseases (Montagnier et al., 1984); high LAV seropositivity among these populations (Brun-Vézinet et al., 1984); a tropism and cytopathic effect in vitro for the helper/inducer T-lymphocyte subset T4 (Klatzmann et al., 1984), also found depleted in vivo.

Other groups have reported the isolation of human retroviruses, the human T cell leukemia/lymphoma/lymphotropic virus type III (HTLV-III) (Popovic et al., 1984) and the AIDS-associated retrovirus (ARV), which display biological and sero-epidemiological properties very similar to if not identical with those of LAV (Levy et al., 1984; Popovic et al., 1984; Schüpbach et al., 1984). Both LAV and HTLV-

III genomes have been molecularly cloned (Alizon et al., 1984; Hahn et al., 1984). Their restriction maps show remarkable agreement, including a Hind III restriction site polymorphism, bearing in mind the variability of this virus (Shaw et al., 1984) and confirming that these two viruses represent a single viral lineage.

In addition to its obvious diagnostic and therapeutic potential, the LAV DNA nucleotide sequence is essential to an understanding of the genetics and molecular biology of the virus and its classification among retroviruses. We report here the complete 9193-nucleotide sequence of the LAV genome established from cloned proviral DNA.

Results

DNA Sequence and Organization of the LAV Genome

We have reported previously the molecular cloning of both cDNA and integrated proviral forms of LAV (Alizon et al., 1984). The recombinant phage clones were isolated from a genomic library of LAV-infected human T-lymphocyte DNA partially digested by Hind III. The insert of recombinant phage λ J19 was generated by Hind III cleavage within the R element of the long terminal repeat (LTR). Thus each extremity of the insert contains one part of the LTR. We have eliminated the possibility of clustered Hind III sites within R by sequencing part of an LAV cDNA clone, pLAV 75 (Alizon et al., 1984), corresponding to this region (data not shown). Thus the total sequence information of the LAV genome can be derived from the λ J19 clone.

Using the M13 shotgun cloning and dideoxy chain termination method (Sanger et al., 1977), we have determined the nucleotide sequence of λ J19 insert. The reconstructed viral genome with two copies of the R sequence is 9193 nucleotides long. The numbering system starts at the cap site (see below) of virion RNA (Figure 1).

The viral (+) strand contains the statutory retroviral genes encoding the core structural proteins (gag), reverse transcriptase (pol), and envelope protein (env), and two extra open reading frames (orf) that we call Q and F (Table 1). The genetic organization of LAV, 5'LTR-gag-pol-Q-env-F-3'LTR, is unique. Whereas in all replication-competent retroviruses pol and env genes overlap, in LAV they are separated by orf Q (192 amino acids) followed by four small (<100 triplets) orf. The orf F (206 amino acids) slightly overlaps the 3' end of env and is remarkable in that it is half-encoded by the U3 region of the LTR.

Such a structure clearly places LAV apart from previously sequenced retroviruses (Figure 2). The (-) strand is apparently noncoding. The additional Hind III site of the LAV clone λ J81 (with respect to λ J19) maps to the apparently noncoding region between Q and env (positions 5166-5745). Starting at position 5501 is a sequence (AAGCCT) that differs by a single base (underlined) from the Hind III recognition sequence. It is anticipated that many of the restriction site polymorphisms between different isolates will map to this region.

[illegible]

LeuGlyIleIleGlnAlaGlnProAspLysSerGluSerGluLeuValAsnGlnIleIleGluGlnLeuIleLysLysGluLysValTyrLeuAlaTrpValProAlaHisLysGlyIle
ATTACCAATCAATCAAGCACAACCCAGATAAAGTCAATCAGAGTTAGTCAATCAATTAAGAGCAGTAAATAAGAGGAAAGGCTCTATCGCATGGGTACCCAGCACAAGCAAT
3700
GlyGlyAsnGlnGlnValAspLysLeuValSerAlaGlyIleArgLysValLeuPheLeuAspGlyIleAspLysAlaGlnAspGluHisGluLysTyrHisSerAsnTrpArgAlaMet
TGGAGGAAATCAACAGTACATATAATAGTCACTGCTGCAATCAGGAAAGTACTATTTTATAGTGAATAGATAGAGCCCAAGATCAACATGAGAAATATCAGATAATTGGAGCAAGT
3800
AlaSerAspPheAsnLeuProProValValAlaLysGluIleValAlaSerCysAspLysCysGlnLeuLysGlyGluAlaMetHisGlyGlnValAspCysSerProGlyIleTrpGln
GGCTAGTCAATTTAACCTGCCACTGTAGTACCAAGAAATAGTAGCCAGCTGTGATAAATGTCAGCTAAAAGGACAAGCCATGCTGACACAGTACAGTCTAGTCCAGGAATATGCCA
3900
LeuAspCysThrHisLeuGluGlyLysValIleLeuValAlaValHisValAlaSerGlyTyrIleGluAlaGluValIleProAlaGluThrGlyGlnGluThrAlaTyrPheLeuLeu
ACTAGATTGTACACATTTAGAACGAAAGTATCTCTGCTAGCAGCTTCATCTAGCCAGTGCATATATAGAACGACAAGTTATTCCAGCAGAAACAGGGCAGAAACAGCACTACTTCTTTT
4000
LysLeuAlaGlyArgTrpProValLysThrIleHisThrAspAsnGlySerAsnPheThrSerThrThrValLysAlaAlaCysTrpTrpAlaGlyIleLysGlnGluPheGlyIlePro
AAAAATAGCAGGAGATGCCAGTAAAAACAATACATACACACAATGCCAGCAATTTCCAGCTACTAGCTTAAGCCCGCTGTGCTGGCCGCAATCAAGCAGCAATTTGCAATTC
4100
TyrAsnProGlnSerGlnGlyValValGluSerMetAsnLysGluLeuLysLysIleIleGlyGlnValArgAspGlnAlaGluHisLeuLysThrAlaValGlnMetAlaPheIle
CTACAATCCCAAGTCAAGGAGTACAGTATCTATGAATAAAGAAATTAAGAAATATAGCCAGTCAAGCTGAACATCTTAAGCAGCAGTACAAATGCAATCTTCTAT
4200
HisAsnPheLysArgLysGlyGlyIleGlyGlyTyrSerAlaGlyGluArgIleValAspIleIleAlaThrAspIleGlnThrLysGluLeuGlnLysGlnIleThrLysIleGlnAsn
CCCAATTTTAAAGAAAGGGGGATTTGGGGTACAGTCCAGGGGAAAGAACTAGTAGACATAATAGCAACAGACATACAACTAAAGAAATACAAAAACAAATACAAAAATTCAAAA
4300
PheArgValTyrTyrArgAspSerArgAspProLeuTrpLysGlyProAlaLysLeuLeuTrpLysGlyGluGlyAlaValValIleGlnAspAsnSerAspIleLysValValProArg
TTTTCGGGTTTATTACAGGACAGCAGACATCCACTTTGGAGAGCAGCAGCAAGCTCTCTGGAAGGCTGAGGGGCACTAGTAATACAGATAATAGTGCATATAAGTACTGCCAAG
4400
ArgLysAlaLysIleIleArgAspTyrGlyLysGlnMetAlaGlyAspAspCysValAlaSerArgGlnAspGluAsp
GluLysGlnArgSerLeuGlyIleMetGluAsnArgTrpGlnValMetIleValTrpGlnValAspArgMetArgIleArgThrTrpLysSerLeuValLysHisHisMetTyrValSer
AAGAAAGCAAGATCATTAGGATATGGAAGACAGTGGCAGGTGATGATGCTGGGCAAGTAGACAGGATGAGGATTAGAACATGGAAGTTAGTAAACACCAATATGATGTTT
4500
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CAGGGAAGCTAGGGATGCTTTATAGACATCACTATGAAGCCCTCATCCAGAAATAGTTTCAGAGTACACATCCCACTAGGGGATGCTAGTTGTAATACAAATATTTGGGCTC
4600
HisThrGlyGluArgAspTrpHisLeuGlyGlnGlyValSerIleGluTrpArgLysLysArgTyrSerThrGlnValAspProGluLeuAlaAspGlnLeuIleHisLysTyrPhe
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4700
AspCysPheSerAspSerAlaIleArgLysAlaLeuLeuGlyHisIleValSerProArgCysGluTyrGlnAlaGlyHisAsnLysValGlySerLeuGlnTyrLeuAlaLeuAlaAla
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4800
LeuIleThrProLysLysIleLysProProLeuProSerValThrLysLeuThrGluAspArgTrpAsnLysProGlnLysThrLysGlyHisArgGlySerHisThrMetAsnGlyHis
CATTAATAACACCAAAAGATAAGCCACCTTTGCTAGTCTTACGAACTCAGACAGGATAGATGCAACAGCCCAAGAGCAAGGCGGACAGAGGAGCCACACCAATGAATGAC
4900
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5000
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5100
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5200
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5300
TACTAGCAATATAATAGCAATAGTCTGTGCTCCATAGTAAATCAGCAATATAGCAAAATATTAAGCAAAAGAAATAGACAGGTTAATGATAGACTAATAGAAAGAGCAGAGACA
5400
ValAlaIleArgValLysGluLysTyrGlnHisLeuTrpArgTrpGlyTrpLysTrpGlyThrMetLeuLeuGlyIleLeuMetIleCysSerAlaThrGluLysLeuTrpValThrVal
GTGCGAATGAGAGTCAAGGAGAAATATCAGCACTTCTGACAGTGGCGGTGAAATGGGGCAGCATGCTCTCTGGATATTGATGATGCTGAGTGTACAGAAATATGCTGGTCAAGTCT
5500
TyrTyrGlyValProValTrpLysGluAlaThrThrLeuPheCysAlaSerAspAlaLysAlaTyrAspThrGluValHisAsnValTrpAlaThrHisAlaCysValProThrAsp
TATTATGGGCTGCTGTGCAAGCAAGCAACCACTCTATTGTCATAGATAGATGCAAGTATATACAGAGTATGTAATGTTGGCCACACATGCTCTGTACCCACAC
5600
ProAsnProGlnGluValLeuValAsnValThrGluAsnPheAsnMetTrpLysAsnAspMetValGluGlnMetHisGluAspIleIleSerLeuTrpAspGlnSerLeuLysPro
CCCAACCCACAAGAGTAGTATTGCTAAATGTCAGCAAAATTTTAACTGTGGAATAATGATGTAAGACAGATGCTAGGATATATAGCTTTATGGATCAAAAGCTTAAAGCCA
5700
CysValLysLeuThrProLeuCysValSerLeuLysCysThrAspLeuGlyAsnAlaThrAsnThrAsnSerSerAsnThrAsnSerSerGlyGluMetMetGluLysGlyGlu
TGTGTAATTAACCCCACTCTGTGTTAGTTTAAAGTCACTGATTGCGGAATGCTACTAATACCAATAGTAGTAATACCAATAGTACTAGCCGGAATGATGATGAGAAAGAGAG
5800
IleLysAsnCysSerPheAsnIleSerThrSerIleArgGlyLysValGlnLysGluTyrAlaPhePheTyrLysLeuAspIleIleProIleAsnAspThrThrSerTyrThrLeu
ATAAAAAGTCTCTTCAATATCAGCACAAGCAATAGAGTCAAGGTCAGCAAGAAATATGCAATTTTATAACTGTATATAATAGCAATAGATATGATAGTACAGCTATACGTTG
5900
ThrSerCysAsnThrSerValIleThrGlnAlaCysProLysValSerPheGluProIleProIleHisTyrCysAlaProAlaGlyPheAlaIleLeuLysCysAsnAsnLysThrPhe
ACAAGTCTTAACACCTCACTATTACACAGGCTGCTCCAAAGGTATCTTTGACCAATTTCCATACATTATTGTCGCGGCTGTTTCCGATTCTAAATGTAATAAGACGCTTC
6000
AsnGlyThrGlyProCysThrAsnValSerThrValGlnCysThrHisGlyIleArgProValValSerThrGlnLeuLeuLeuAsnGlySerLeuAlaGluGluValValIleArg
AATGGAACAGGACCATGTACAAATGTCAGCAGTACAAATGTCAGTACGAAATAGCCAGTACTTCAACTCACTCTCTGCAATGCGCTGACCAAGCAAGAGCTAGTAAATAGA
6100
SerAlaAsnPheThrAspAsnAlaLysThrIleIleValGlnLeuAsnGlnSerValGluIleAsnCysThrArgProAsnAsnAsnThrArgLysSerIleArgIleGlnArgGlyPro
TCTGCCAATTTTACACAGCAATGCTAAAGCATTAATAGTACAGCTGAACCAATCTGTACAAATTAATGTAAGAGCCCAACAAATACAGCAAAAGTATCCGTATCCAGAGGGACCA
6200
GlyArgAlaPheValThrIleGlyLysIleGlyAsnMetArgGlnAlaHisCysAsnIleSerArgAlaLysTrpAsnAlaThrLeuLysGlnIleAlaSerLysLeuArgGluGlnPhe
GGCAGAGCATTTGTTACAATAGCAAAATAGCAATATGACAGCAGCATTTGTAAGATTAGTACAGCAAAATGCAATGCACTTTAAACACAGTACCTAGCAATTAAGACAACTTT
6300
GlyAsnAsnLysThrIleIlePheLysGlnSerSerGlyGlyAspProGluIleValThrHisSerPheAsnCysGlyGlyGluPhePheTyrCysAsnSerThrGlnLeuPheAsnSer
GCAATATAAAGCAATATCTTAAAGCAATCTCAGGAGGGGAGCCAGCAATTTGTAAGCAGCACTTTTAAATGTTGGAGGGGAATTTTCTACTGTAATTAACACAGTCTTTTATAGT
6400
ThrTrpPheAsnSerThrTrpSerThrGluGlySerAsnAsnThrGluGlySerAspThrIleThrLeuProCysArgIleLysGlnPheIleAsnMetTrpGlnGluValGlyLysAla
ACTTGCTTAAATAGTACTTGCAGTACTGAAGGCTCAAAATACACTGAAGGAGTCAACAAATCAGCTCCATGCCAGCAATAAAGCAATTTAATCAATGTCGAGCAAGTACGAAAGCA
6500
MetTyrAlaProProIleSerGlyGlnIleArgCysSerAsnIleThrGlyLeuLeuLeuThrArgAspGlyGlyAsnAsnAsnGlySerGluIlePheArgProGlyGlyGly
ATGTATGCCCTCCCATCAGCGGCAAAATAGATGTTCAATCAATATACAGGCTGCTATTAACAGAGATGCTGCTAATAACAACTAGCTGCTGAGATCTTCAGACTCTGAGCAGCA
6600
AspMetArgAspAsnTrpArgSerGluLeuTyrLysTyrLysValValLysIleGluProLeuGlyValAlaProThrLysAlaLysArgValValGlnArgGluLysArgAlaVal
GATATGAGGCAATGTCAGAGTCAATTAATAATATAAGTACTAAAAATTAACCATAGCAGTACGACCCAGCAAGGCAAGAGCAAGAGTGTGTCAGAGCAAAAGAGCAGTGT
6700

Figure 1. Complete DNA Sequence of Viral Genome (LAV-1a)

Each nucleotide was sequenced on average 5.3 times: 85% of the sequence was determined on both strands and the remainder was sequenced at least twice from independent clones. The base composition is T, 22.2%; C, 17.8%; A, 35.8%; G, 24.2%; G + C, 42%. The dinucleotide CpG is greatly under-represented (0.9%) as is common among eukaryotic sequences (Bird, 1980).

The LTR
The organization of a reconstructed LTR and viral flanking elements are shown schematically in Figure 3. The LTR is 638 bp long and displays usual features (Chen and Barker, 1984): it is bounded by an inverted repeat (5'ACTG) including the conserved TG dinucleotide (Temin, 1981); adjacent to 5' LTR is the tRNA primer binding site (PBS), complementary to tRNA^{lys} (Raba et al., 1979); adjacent to 3' LTR is a perfect 15 bp polypurine tract. The other three

The limits of U5, R, and U3 elements were determined as follows. U5 is located between PBS and the polyadenylation site established from the sequence of the 3' end of oligo(dT)-primed LAV cDNA (Alizon et al., 1984). Thus U5 is 84 bp long. The length of R+U5 was determined by synthesizing tRNA-primed LAV cDNA. After alkaline hydroly-

orf	1 st Triplet	Met	Stop	No. Amino Acids	M, Calc.
				500	55,841
gag	312	336	1,836	(1,003)	(113,629)
pol	1,631	1,934	4,640		22,487
orf Q	4,554	4,587	5,163	192	97,376
env	5,746	5,767	8,350	861	23,316
orf F	8,324	8,354	8,972	206	

The nucleotide coordinates refer to the first base of the first triplet (1st triplet), of the first methionine (initiation) codon (Met) and of the stop codon (Stop). The numbers of amino acids and molecular weights are those calculated for unmodified precursor products starting at the first methionine through to the end, with the exception of pol, where the size and M_r refer to that of the whole orf.

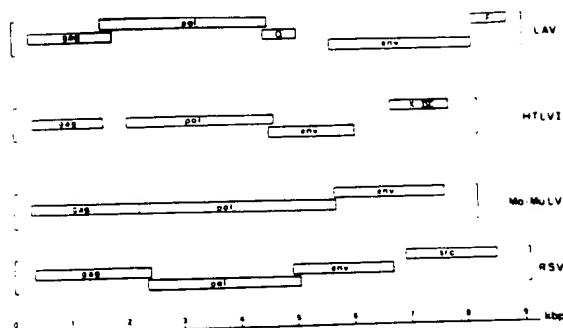


Figure 2. Comparison of the Genome Organization of LAV with Those of Human T Cell Leukemia/Lymphoma Virus Type I (HTLV-I) (Seiki et al., 1983), Moloney Murine Leukemia Virus (MoMuLV) (Shinnick et al., 1981), and Rous Sarcoma Virus (RSV) (Schwartz et al., 1983)

The positions and sizes of viral genes are drawn to scale (open boxes) and the viral genomes (RNA forms) are delimited by brackets.

sis of the primer, R+U5 was found to be 181 ± 1 bp (Figure 4). Thus R is 97 bp long and the cap site at its 5' end can be located. Finally, U3 is 456 bp long. The LAV LTR also contains characteristic regulatory elements: a polyadenylation signal sequence AATAAA 19 bp from the R-U5 junction, and the sequence ATATAAG, which is very likely the TATA box, 22 bp 5' of the cap site. There are no long direct repeats within the LTR. Interestingly, the LAV LTR shows some similarities to that of the mouse mammary tumor virus (MMTV) (Donehower et al., 1981). They both use tRNA^{phe} as a primer for (-) strand synthesis, whereas all other exogenous mammalian retroviruses known to date use tRNA^{pro} (Chen and Barker, 1984). They possess very similar polypurine tracts; that of LAV is AAAAGAAAGGGGGG while that of MMTV is AAAAAAGAAAAAGGGGG. It is probable that the viral (+) strand synthesis is discontinuous since the polypurine tract flanking the U3 element of the 3'LTR is found exactly duplicated in the 3' end of orf pol, at 4331-4346. In addition, MMTV and LAV are exceptional in that the U3 element can encode an orf. In the case of MMTV, U3 contains the whole orf while, in LAV, U3 contains 110 codons of the 3' half of orf F.

Viral Proteins

gag

Near the 5' extremity of the gag orf is a "typical" initiation codon (Kozak, 1984) (position 336), which is not only the first in the gag orf, but the first from the cap site. The precursor protein is 500 amino acids long. The calculated M_r of 55,841 agrees with the 55 kd gag precursor polypeptide (Luc Montagnier, unpublished results). The N-terminal amino acid sequence of the major core protein p25, obtained by microsequencing (Genetic Systems, personal communication), matches perfectly with the translated nucleotide sequence starting from position 732 (see Figure 1). This formally makes the link between the cloned LAV genome and the immunologically characterized LAV p25 protein. The protein encoded 5' of the p25 coding sequence is rather hydrophilic. Its calculated M_r of 14,866 is consistent with that of the gag protein p18. The 3' part of the gag region probably codes for the retroviral nucleic acid binding protein (NBP). Indeed, as in HTLV-I (Seiki et

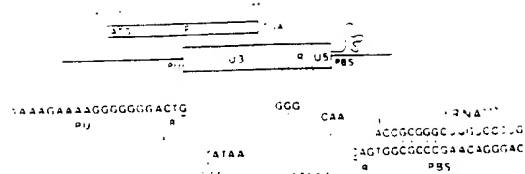


Figure 3. Schematic Representation of the LAV Long Terminal Repeat (LTR)

The LTR was reconstructed from the sequence of UJ19 by juxtaposing the sequences adjacent to the Hind III cloning sites. Sequencing of oligo(dT)-primed LAV DNA clone pLAV75 (Alizon et al., 1984) rules out the possibility of clustered Hind III sites in the R region of LAV. LTR are limited by an inverted repeat sequence (IR). Both of the viral elements flanking the LTR have been represented as tRNA primer binding site (PBS) for 5' LTR and polypurine tract (PU) for 3' LTR. Also indicated are a putative TATA box, the cap site, polyadenylation signal (AATAAA), and polyadenylation site (CAA). The location of the open reading frame F (648 nucleotides) is shown above the LTR scheme.

al., 1983) and RSV (Schwartz et al., 1983), the motif Cys-X₂-Cys-X₄₋₆-Cys common to all NBP (Oroszlan et al., 1984) is found duplicated (nucleotides 1509 and 1572 in LAV sequence). Consistent with its function the putative NBP is extremely basic (17% Arg + Lys).

pol

The reverse transcriptase gene can encode a protein of up to 1003 amino acids (calculated M_r = 113,629). Since the first methionine codon is 92 triplets from the origin of the open reading frame, it is possible that the protein is translated from a spliced messenger RNA, giving a gag-pol polyprotein precursor.

The pol coding region is the only one in which significant homology has been found with other retroviral protein sequences, three domains of homology being apparent. The first is a very short region of 17 amino acids (starting at 1856). Homologous regions are located within the p15 gag^{RSV} protease (Dittmar and Moelling, 1978) and a polypeptide encoded by an open reading frame located between gag and pol of HTLV-I (Figure 5) (Schwartz et al., 1983; Seiki et al., 1983). This first domain could thus correspond to a conserved sequence in viral proteases. Its different locations within the three genomes may not be significant since retroviruses, by splicing or other mechanisms, express a gag-pol polyprotein precursor (Schwartz et al., 1983; Seiki et al., 1983). The second and most extensive region of homology (starting at 2048) probably represents the core sequence of the reverse transcriptase. Over a region of 250 amino acids, with only minimal insertions or deletions, LAV shows 38% amino acid identity with RSV, 25% with HTLV-I, and 21% with MoMuLV (Schinnick et al., 1981) while HTLV-I and RSV show 38% identity in the same region. A third homologous region is situated at the 3' end of the pol reading frame and corresponds to part of the pp32 peptide of RSV that has exonuclease activity (Misra et al., 1982). Once again, there is greater homology with the corresponding RSV sequence than with HTLV-I.

env

The env open reading frame has a possible initiator methionine codon very near the beginning (eighth triplet).

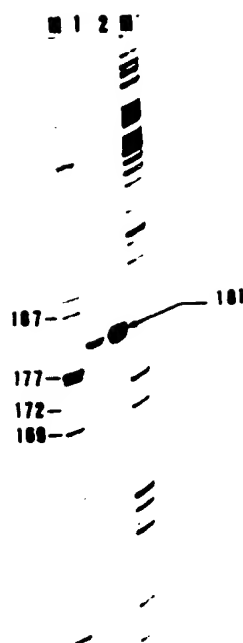


Figure 4. Synthesis of RNA-Primed LAV cDNA for R+U5 (Strong-Stop cDNA)

Lanes 1 and 2 show two different quantities of cDNA while lanes M and M' represent markers. The strong-stop cDNA is 181 bases long with a second, less intense band at 180. The error of estimation is ± 1 bp. This maps the major cap site to the second G residue of the sequence CTGGGTCT within the LTR, 24 nucleotides downstream of the TATA box. This guanosine residue is taken as the first base in the nucleotide sequence shown in Figure 1.

If so, the molecular weight of the presumed env precursor protein (861 amino acids, M_r calc = 97,376) is consistent with the known size of the LAV glycoprotein (110 kd and 90 kd after glycosidase treatment; Luc Montagnier, unpublished). There are 32 potential N-glycosylation sites (Asn-X-Ser/Thr), which are overlined in Figure 1. An interesting feature of env is the very high number of Trp residues at both ends of the protein. There are three hydrophobic regions, characteristic of the retroviral envelope proteins (Seiki et al., 1983), corresponding to a signal peptide (encoded by nucleotides 5815–5850 bp), a second region (7315–7350 bp), and a transmembrane segment (7831–7896 bp). The second hydrophobic region (7315–7350 bp) is preceded by a stretch rich in Arg + Lys. It is possible that this represents a site of proteolytic cleavage, which, by analogy with other retroviral proteins, would give an external envelope polypeptide and a membrane-associated protein (Seiki et al., 1983; Kiyokawa et al., 1984). A striking feature of the LAV envelope protein sequence is that the region following the transmembrane segment is of unusual length (150 residues). The env protein shows no

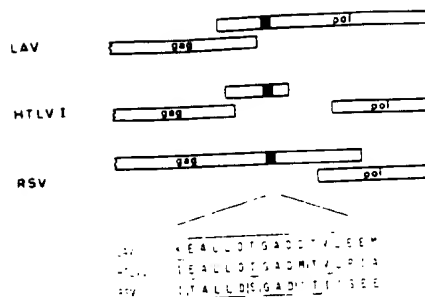


Figure 5. Location of a Short Stretch of Homology in the gag-pol Region of the LAV, HTLV-I (Seiki et al., 1983) and RSV (Schwartz et al., 1983) Genomes

Conserved amino acids are boxed. Homologous region is shown by the solid bar in the schema. Each virus is organized differently in this region but the sequence in the RSV genome maps to p15^{gag}, which has a protease-associated function.

homology to any sequence in protein data banks. The small amino acid motif common to the transmembrane proteins of all leukemogenic retroviruses (Ciinciolo et al., 1984) is not present in LAV env.

Q and F

The location of orf Q is without precedent in the structure of retroviruses. Orf F is unique in that it is half-encoded by the U3 element of the LTR. Both orf have strong initiator codons (Kozak, 1984) near their 5' ends and can encode proteins of 192 amino acids (M_r calc = 22,487) and 206 amino acids (M_r calc = 23,316), respectively. Both putative proteins are hydrophilic (pQ 49% polar, 15.1% Arg + Lys; pF 46% polar, 11% Arg + Lys) and are therefore unlikely to be associated directly with membrane. The function for the putative proteins pQ and pF cannot be predicted, as no homology was found by screening protein sequence data banks. Between orf F and the pX protein of HTLV-I there is no detectable homology. Furthermore, their hydrophobicity/hydrophilicity profiles are completely different. It is known that retroviruses can transduce cellular genes—notably proto-oncogenes (Weinberg, 1982). We suggest that orfs Q and F represent exogenous genetic material and not some vestige of cellular DNA because LAV DNA does not hybridize to the human genome under stringent conditions (Alizon et al., 1984), and their codon usage is comparable to that of the gag, pol, and env genes (data not shown).

Relationship to Other Retroviruses

Although LAV is both morphologically and biochemically (Barré-Sinoussi et al., 1983) distinct to HTLV-I and -II, it remained possible that its genome was organized in a similar manner. The characteristic features of HTLV-I and -II genomes, which they share with the more distantly related bovine leukemia virus (BLV) (Rice et al., 1984), are not observed in the case of LAV. These are: a region 3' of the envelope gene consisting of a noncoding stretch (600–900 bp), followed by a coding sequence of 307–357 codons (X open reading frame), which may slightly overlap the U3 region of the LTR (Seiki et al., 1983; Rice et al., 1984; Sagata et al., 1984) and, second, the LTR being

Table 2. Comparison of the Size of the LAV LTR and LTR-Related Element to Those of Other Retroviruses

	LTR	U3	R	U5	PU	PBS	IR
LAV	638	456	97	85	15	LYS	4
HTLV-I	759	355	228	176	12'	PRO	4'
HTLV-II	763	314	248	261	12'	PRO	4'
MMTV	1,332	1,197	11	124	19	LYS	8'
MoMuLV	594	449	68	77	13	PRO	13
RSV	335	234	21	80	11	TRP	15
SNV	601	420	97	80	13	PRO	9

Adapted from Chen and Barker (1984).

i = imperfect match or tract.

SNV = spleen necrosis virus (Shimotohno and Temin, 1982).

composed of unusually long U5 and R elements and the polyadenylation signal being situated in U3 instead of R (Seiki et al., 1983; Sagata et al., 1984; Shimotohno et al., 1984). We show here that, in contrast, the 3' end of the LAV envelope gene overlaps an open reading frame, termed F, that has the coding capacity for 206 amino acids and extends within the LTR (110 amino acids are encoded by the U3 region). The putatively encoded polypeptide (pF), the primary structure of which can be deduced, does not show any homology with the theoretical X gene products of the HTLV/BLV family. Also, the U5 and R elements are shorter (Table 2) and the polyadenylation signal is located within R, as is the case for all retroviruses except the HTLV/BLV. Additionally, LAV uses tRNA^{lys} as (-) strand primer, as opposed to tRNA^{pro} employed by all other mammalian retroviruses except MMTV (Donehower et al., 1981). Those homologies detected between the polymerase and protease domains of LAV and HTLV are also found in several retroviruses, RSV in particular.

It has been reported that a cloned HTLV-III genome hybridizes ($T_m = 28^\circ\text{C}$) to sequences in the gag-pol and X regions of HTLV-I and -II; although restriction maps of cloned LAV and HTLV-III show almost perfect agreement (Hahn et al., 1984), we were unable to detect any such hybridization between LAV and HTLV-II ($T_m = 55^\circ\text{C}$) (Alizon et al., 1984). Indeed, there is a punctual region of homology between LAV and HTLV-I (23/27' nucleotides starting at position 1859 in the LAV sequence) but nothing significant between the two viruses in the X region of HTLV-I. One possible reason for this discrepancy is that HTLV-III is subtly different from LAV. However it was subsequently reported that there was very minimal, if any, homology between orf X (of HTLV-I) and HTLV-III (Shaw et al., 1984).

Discussion

Regulatory sequences carried by retroviral LTR are believed to be involved in specific interactions between the viral genome and the host cell (Srinivasan et al., 1984). The LTR sequences of LAV are unique among retroviruses. That could reflect an original mode of gene expression, possibly in relation to particular transcriptional factors present in the virus-harboring cell. This hypothesis can be tested by studying the regulatory activity of the LAV

LTR sequences in transient or long-term experiments involving an indicator gene and different cellular contexts.

The presence of the Q and F reading frames in addition to the conventional gag-pol-env set of genes is unexpected. One should now address the question of their role in the viral cycle and pathogenicity by trying to characterize their protein product(s). It is tempting to speculate on a role of such polypeptide(s) in T4 cells' mortality, a problem that can be studied by designing synthetic peptides for antibody production or by using site-directed mutagenesis of Q and F coding regions.

The peculiar genetic structure of LAV poses the question of its origin. The virus shares common tracts with other (apparently unrelated) retroviruses. For instance, the unusually large size of the outer membrane glycoprotein (env) and a comparably sized genome are also observed in the case of lentiviruses such as Visna (Harris et al., 1981; Querat et al., 1984). The presence of a large part of the F open reading frame in the LTR, and the use of tRNA^{lys} as a primer for (-) strand synthesis, is reminiscent of the mouse mammary tumor virus. On the other hand, homologies in the pol gene would suggest that the LAV is closer to RSV than to any other retroviruses. Obviously, no clear picture can be drawn from the DNA sequence analysis as far as phylogeny is concerned. Thus, it may well be that LAV defines a new group of retroviruses that have been independently evolving for a considerable period of time, and not simply a variant recently derived from a characterized viral family. Both epidemiology and pathogeny of AIDS should be reconsidered with this idea in mind, when trying to answer such questions as these: Are there other human or animal diseases that are associated with similarly organized viruses? Is there a precursor to AIDS-associated virus(es) normally present, in latent form, in human populations? What triggered in this case the recent spreading of pathogenic derivatives?

Experimental Procedures

M13 Cloning and Sequencing

Total L19 DNA was sonicated, treated with the Klenow fragment of DNA polymerase plus deoxyribonucleotides (2 hr, 16°C), and fractionated by agarose gel electrophoresis. Fragments of 300–600 bp were excised, electroeluted, and purified by Elutip (Schleicher and Schüll) chromatography. DNA was ethanol-precipitated using 10 μg dextran T40 (Pharmacia) as carrier and ligated to dephosphorylated, Sma I-cleaved M13mp8 RF DNA using T4 DNA and RNA ligases (16 hr, 16°C) and transfected into E. coli strain TG-1. Recombinant clones were detected by plaque hybridization using the appropriate ³²P-labeled LAV restriction fragments as probes. Single-stranded templates were prepared from plaques exhibiting positive hybridization signals and were sequenced by the dideoxy chain termination procedure (Sanger et al., 1977) using α -³²S-dATP (Amersham, 400 Ci/mmol) and buffer gradient gels (Biggen et al., 1983). Sequences were compiled and analyzed using the programs of Staden adapted by B. Caudron for the Institut Pasteur Computer Center (Staden, 1982).

Strong-Stop cDNA

LAV virions from infected T lymphocyte (Barre-Sinoussi et al., 1983) culture supernatant were pelleted through a 20% sucrose cushion and the cDNA (-) strand was synthesized as described previously (Alizon et al., 1984) except that no exogenous primer was used. After alkaline hydrolysis (0.3 M NaOH, 30 min, 65°C), neutralization, and phenol extraction, the cDNA was ethanol-precipitated and loaded onto a 6%

acrylamide/8 M urea sequencing gel with sequence ladders as size markers.

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Molecular turnover and memory

SIR — Francis Crick considers in *News and Views* under the title "Memory and molecular turnover" the problem, often overlooked, that although memory operates over periods of years or decades, most macromolecules (with the exception of DNA) turn over with half lives of hours or weeks. Crick sees the dilemma since memory is prolonged and a consequence of inter-synaptic interaction which is dependent on fixed intrasynaptic macromolecules, such as membrane glycoproteins, either singly or more probably in larger aggregates of some form. To sustain memory two alternative strategies are proposed, either the memory macromolecule is immune from turnover (a less likely possibility for Crick) or the memory macromolecules in a synapse can be replaced one at a time without altering the overall state of the memory macromolecular complex.

My response to Crick's interesting challenge lies in the observation that perinuclear (often sided) membrane disposition is required *before* protein catabolism in normal cells takes place² and some special association of proteins with cytoskeletal elements may precede routing to the cellular destructive machinery^{3,4}.

Nerve cells are exquisitely polarized with the cell body (nucleus, polysomes and Golgi) quite spatially distinct from synapse forming processes such as axons or dendrites. Therefore, memory proteins dispatched from around the nucleus into processes by axoplasmic flow would need to retrace their steps for destruction as described in the protein turnover cycle⁵. It is not at all fanciful to suppose that the intricate arborizations of nerve cell processes in the temporal cortex (and everywhere else) have evolved, at least in part, in order to separate and 'immunize' informational macromolecules spatially from the apparatus of molecular turnover. Simply detaching such macromolecules from the neuroskeletal system would suffice (compare refs 2-4), thereby preventing (or slowing) the return of the macromolecules to the perinuclear destructive machinery. Alternatively, selective reversible detachment-attachment to the neuroskeleton would identify populations of proteins (or individual proteins) which are to be routed for destruction and replacement.

Both of the above alternatives could be mediated by protein modifications such as those described by Crick. Likewise both possibilities could operate together in a single neurone. Either way, neuronal evolution could have capitalized on the spatial separation of proteins (in membranes) in the synapses of cellular processes away from the perinuclear destruction apparatus in cell bodies so that information storage is achieved by permanent or temporary macromolecular stabilization.

It is perhaps salutary in an ageing human

population that facultative loss, as occurs in Alzheimer's disease, and motor disorder, as seen in Parkinson's disease, might be triggered by the disruption of the status of cell body-synaptic trafficking in cortical neurones and nigro-striated pathways respectively, leading to intraneuronal degradative (degenerative) processes.

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Human B-cell cytotoxic lymphokine priority

SIR — The recent article on the cloning and expression of human lymphotoxin by scientists from Genentech Inc.¹ described work that first came to my attention when a report appeared in *The Guardian* of 6 June 1984 concerning an announcement by the company that they had developed a new cancer drug which did not have side effects. The initial scientific papers from Genentech² stated that the lymphotoxin in question had a relative molecular mass (M_r) of approximately 20,000. I therefore thought that it must be a new cytotoxic lymphokine. Some seven years earlier^{3,4}, I had published a description of a humoral cytotoxic factor produced by a human lymphoblastoid cell line of B-cell lineage derived from a local patient with leukaemia.

Those papers of mine, which characterized the properties of the factor and how it was distinguished from other forms of cell killing, were probably the first well documented studies showing that some human B-lymphoblasts growing *in vitro* produced a cytotoxic lymphokine. In these early papers we also reported that the humoral factor preferentially kills malignant cells and that it had reduced by approximately 50 per cent the incidence of malignancy (fibrosarcoma) in mice^{3,4}. Our studies also showed that the cytotoxic factor was a protein with an M_r of $65,000 \pm 1,000^5$.

In the recent paper in *Nature* by Genentech, the authors state that their initial published result (1984) on the relative molecular mass was incorrect and that the actual M_r of this human lymphoblastoid cell-derived lymphotoxin is 60,000-70,000. This value is so closely similar to the 65,000 M_r we had previously reported in 1980³, that it now seems extremely likely that the human B-lymphoblast-produced lympho-

toxin — cloned recently by Genentech — is identical with the cytotoxic factor first described in 1977^{1,4}. It will be for future studies to establish whether the cytotoxic factor described from Cambridge in 1977 and the lymphotoxin described in 1984 in the United States are in fact identical and to explore the efficacy in the treatment of malignant states.

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HTLV-III, LAV, ARV are variants of same AIDS virus

SIR — Retroviruses have been isolated reproducibly from patients with the acquired immunodeficiency syndrome (AIDS), and have been designated human T-cell lymphotropic virus (HTLV) type III¹, lymphadenopathy-associated virus (LAV)², or rather recently AIDS-related virus (ARV)³ by different groups of investigators. Forty-eight independent HTLV-III isolates were originally reported from our laboratory¹, several additional ones since^{4,5}, and now we have obtained more than 100 independent isolates (S.Z. Sakhuddin *et al.*, in preparation). The recent publications of the complete nucleotide sequence of two HTLV-III proviruses⁶, LAV⁷, and ARV⁸ allows a detailed comparison (see table). Sequences of HTLV-III clones BH5 and BH8 (representing the 5' and 3' portions of provirus(es), respectively), clone LAV1a, and ARV-2 are compared to HTLV-III clone BH10. LAV is closely related to HTLV-III clone BH10 and differs in 1.5% of nucleotides and 2.2% of amino acids, while ARV-2 differs in 6.3% of its base pairs and 9.2% of its amino acids from that of HTLV-III clone BH10. These data show that HTLV-III, LAV, and ARV are variants of the same virus. The greater sequence divergence of ARV from HTLV-III is not likely to be a result of errors in sequence determination. First, sequences obtained independently in different laboratories for the same HTLV-III clones were in agreement⁴. Second, multiple clones of ARV isolated from the same cell line infected with a virus isolate from a single individual differ in sequence from one another by only 2 or 3 base pairs (bp)⁸. Third, we have sequenced another proviral clone of HTLV-III derived from another one (RF) of the original 48 isolates reported¹, which differs from BH10 to a similar degree as does ARV (our unpublished observations with B. Starck, B. Hahn

AIDS virus sequences

No. and % (in parentheses) of differences compared with HTLV-III clone BH10 sequence

	HTLV-III clones BH5/BH8					LAV			ARV		
	Total nucleotides	Total amino acids	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*	Nucleotide differences	Amino acid differences	Non-conservative amino acid differences*
LTR†	634	—	3† (1.6)	—	—	10 (1.6)	—	—	30 (4.7)	—	—
Leader and (RNA PBS)‡	152	—	4† (3.6)	—	—	5 (3.3)	—	—	14 (9.2)	—	—
<i>gag</i>	1,536	512	19 (1.2)	7 (1.4)	3 (0.6)	46‡ (3.0)	16‡ (3.1)	2 (0.4)	86‡ (5.6)	32‡ (6.3)	8 (1.6)
<i>pol</i>	3,045	1,015	29 (0.9)	12 (1.1)	3 (0.3)	59‡ (1.9)	21‡ (2.1)	5 (0.5)	134‡ (4.4)	51‡ (5.0)	14 (1.4)
<i>env</i>	609	203	6 (1.0)	4 (2.0)	1 (0.5)	2 (3.3)	0 (0.0)	0 (0.0)	31 (5.1)	20 (9.8)	8 (3.9)
Between <i>env</i> and <i>env-lor</i>	584	—	16 (2.7)	—	—	11 (1.9)	—	—	49 (8.4)	—	—
<i>env-lor</i> :											
Signal peptide	111	37	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.8)	2 (5.4)	0 (0.0)	26§ (23.4)	12§ (32.4)	3 (8.1)
Extracellular portion	1,443	481	27 (1.9)	12 (2.5)	5 (1.0)	32¶ (2.2)	14¶ (2.9)	6 (1.2)	164¶ (11.4)	82¶ (17.0)	41 (8.5)
Transmembrane portion	1,035	345	9 (0.9)	6 (1.7)	2 (0.6)	9 (0.9)	5 (1.4)	2 (0.6)	66 (6.3)	42 (12.2)	19 (5.5)
3' <i>orf</i> **	648	216	12 (1.8)	5 (2.3)	1 (0.5)	13 (2.0)	8 (3.7)	3 (1.4)	52†† (8.0)	29†† (13.4)	10 (4.6)
Total	9,213	2,593	122 (1.3)	40 (1.5)	14 (0.5)	144 (1.5)	58 (2.2)	15 (6.3)	582 (6.3)	239 (9.2)	93

* Considered conservative substitution if same charge or both neutral, and both either hydrophilic or hydrophobic amino-acid insertions and deletions not counted.

† Portion of R, U5 and leader sequence deleted from unintegrated HTLV-III clones BH5, BH8 and BH10, and determined from integrated clone HXB2.

‡ Deletion of one copy of 36-bp (12 amino acid) direct repeat sequence.

§ Insertion of 9-bp sequence and deletion of 15-bp sequence.

|| Deletion of 15-bp (5 amino acid) sequence.

¶ Insertion of 15-bp (5 amino acid) sequence.

§ Deletion of 18-bp (6 amino acid) sequence, insertion of 12 bp (4 amino acid) sequence, deletion of 6 bp (2 amino acid) and 15 bp (5 amino acid) sequences.

** Includes full open reading frame defined by BH8 and LAV sequences: BH10 has a termination codon at amino acid position 134.

†† Insertion of 12-bp (4 amino acid) sequence.

and G. Shaw).

The DNA sequence data confirm the findings from restriction enzyme site analysis of the HTLV-III genomes which show a spectrum of diversity from closely related to more distantly related variants (ref. 9 and F.W.S. *et al.*, unpublished). The closer similarity of the LAV DNA sequence to that of HTLV-III might be because the individuals from whom these isolates were derived acquired the virus at a similar time and place. In fact, many of our earliest HTLV-III isolates were all from specimens obtained in late 1982 or early 1983 from the east coast of the United States^{1,10} and LAV, although isolated from a French man with a lymphadenopathy syndrome, had his contact in New York in the same period². In contrast, the individual from whom ARV was derived was from California, and the specimen was apparently obtained in 1984³. The other more divergent virus (RF) was obtained from a Haitian patient in 1983.

A comparison of the ARV sequence with that of HTLV-III reveals the greatest nucleotide sequence conservation in the LTR and the *gag*, *pol*, and short open reading frame (*sor*) genes. Many of the differences between the AIDS virus sequences represent in-frame deletions and insertions (see notes at bottom of table). The non-coding areas are somewhat less conserved. The most divergence, however, is within the open reading frame,

designated *env-lor*, which encodes the precursor to the envelope proteins and possibly a second protein analogous to *lor* in HTLV-I, HTLV-II, and bovine leukaemia virus (BLV). Of note is the high level of heterogeneity in the extracellular portion of the envelope which differs in 17.0% of its predicted amino acids (8.5% non-conservative amino acid differences) from that predicted for HTLV-III clone BH10. These data may have significant implications for immune system interactions with AIDS virus infected cells, as well as in the design of reagents for viral detection and treatment.

The sequence of clone H9p22 also derived from HTLV-III-infected H9 cells shows 0.5% nucleotide and 0.6% amino-acid differences from that of clone BH10 (ref. 11).

The presence of a *trans*-acting and transcriptional enhancer in HTLV-III-infected cells, as was demonstrated in HTLV-I, HTLV-II and BLV-infected cells, suggests that there is a gene for *lor* in the HTLV-III genome that may mediate this activity¹². We have hypothesized that the 3' portion of the open reading frame designated *env-lor* may be responsible for this function⁸, though others have not made a similar interpretation^{7,8,11}.

The similarities and differences of the AIDS virus isolates to other retroviruses, including the HTLV-BLV family, are discussed in the recent publications⁸⁻¹¹. The

important general structural features of this virus, most notably juxtaposition of myrgaggin, p17, and largaggin, p24 (see ref. 13 for terminology) and the presence of a *lor* coding sequence, suggest that these viruses are related to other members of the HTLV-BLV family. In addition, we are confident that HTLV-III shares nucleotide sequence homology with the *maedi-visna* lenti-retrovirus¹⁴, although another report shows no homology of LAV to *visna*¹⁵. We cannot explain this discrepancy. A more comprehensive discussion of factors to be considered in taxonomic assignments for this virus is forthcoming (W.A. Haseltine *et al.*, in preparation).

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1964-1970

Exhibit 4

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[illegible]

HS⁻ results in a change of only about 20 mV in the corresponding computed Eh in Fig. 2, whereas the total vertical range exceeds 1000 mV. The same argument applies to the computed activity coefficients; any realistic change in activity coefficients results in a trivial change in the distribution of computed Eh values.

The pH is important in determining the distribution of points in Fig. 2. In fact, the linear correlation among computed pE (negative logarithm of the electron activity) (or Eh) values reported in some publications, for example, (8), for coexisting aqueous couples appears to reflect an autocorrelation among the dominant pH terms in the Nernst equations for each of the couples.

There are published suggestions that Eh measurements might be improved if the values were computed from analyses of "indicator" couples, such as I⁻/IO₃⁻ (15) or As(III)/As(V) (16). Unfortunately, this approach does not circumvent the fundamental difficulty that redox reactions in the waters are not at internal equilibrium among themselves. Therefore, an "indicator" Eh no more represents a master redox value for the water than the usual Eh as measured by a noble-metal electrode. Whitfield (4) has suggested that Eh measurements may still be useful as qualitative indicators of the overall redox state of a water sample. However, we believe that it would be better to measure certain sensitive species, such as aqueous oxygen, hydrogen sulfide, or methane as qualitative guides to the redox status of the waters.

The concept of Eh remains a valuable tool for theoretical and pedantic purposes. However, in the apparent absence of internal redox equilibrium, investigators should abandon the use of any measured master Eh for predicting the equilibrium chemistry of redox reactions in normal ground waters. Our conclusions are most severe in the context of predictive computer modeling of the chemistry of natural waters and wastewaters. In order to provide meaningful redox input for such models, it may be necessary to analyze the samples for the dominant ions of every redox element of interest. Wolery (17) has suggested this approach for testing the state of redox equilibrium, using his EQ3NR computer model. If any measured Eh is used as input for equilibrium calculations, the burden rests with the investigator to demonstrate the reversibility of the system.

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Homology of Genome of AIDS-Associated Virus with Genomes of Human T-Cell Leukemia Viruses

Abstract. A T lymphotropic virus found in patients with the acquired immune deficiency syndrome (AIDS) or lymphadenopathy syndrome has been postulated to be the cause of AIDS. Immunological analysis of this retrovirus and its biological properties suggest that it is a member of the family of human T-lymphotropic retroviruses known as HTLV. Accordingly, it has been named HTLV-III. In the present report it is shown by nucleic acid hybridization that sequences of the genome of HTLV-III are homologous to the structural genes (gag, pol, and env) of both HTLV-I and HTLV-II and to a potential coding region called pX located between the env gene and the long terminal repeating sequence that is unique to the HTLV family of retroviruses.

Human T-cell leukemia virus (HTLV) was first identified as an infectious agent etiologically associated with adult T-cell leukemia (ATL) (1). A related but distinct retrovirus was isolated from a T-cell variant of hairy cell leukemia (2). These viruses, known, respectively, as HTLV-I and HTLV-II, show a tropism for human T cells, particularly OKT4⁺ cells, and have the capacity to immortalize and transform normal T cells in culture (3), alter certain T-cell immune functions in vitro (4), induce the formation of giant multinucleated T cells (5), and, in some cases, selectively kill certain T cells (6). These properties and data from epidemiologic studies of the acquired immune deficiency syndrome (AIDS), which is uniformly associated with OKT4⁺ helper cell depletion (7), led us and others to speculate (8) that a member of the HTLV family might be the etiologic agent of this disease. In support of this hypothesis was the finding that up to 80 percent of AIDS patients, but less than 1 percent of non-

AIDS patients from similar risk groups, have serum antibodies that react with the envelope protein of HTLV (9). However, actual isolations of the known subgroups of HTLV (that is, HTLV-I and HTLV-II) from AIDS patients were infrequent (10).

Recently, we reported repeated isolations of a T lymphotropic retrovirus with cytopathic but not immortalizing activity from patients with AIDS (11). This virus can be grown in a previously immortalized T-cell line (HT) that is relatively resistant to the cytopathic effects of the virus and can grow in the absence of T-cell growth factor (interleukin-2) (12). Using the infected cells as well as purified virus particles in immunological assays, we found that the serum of 80 to 100 percent of AIDS patients and 70 to 80 percent of patients with lymphadenopathy syndrome reacted positively (13). On the basis of its T-cell tropism, the size and Mg²⁺ preference of its reverse transcriptase, the size of its major core protein (24,000 daltons) (14), some anti-

genic cross-reactivity of its product with HTLV-I and HTLV-II (14), and its capacity to induce formation of giant multinucleated cells (12), we considered this virus to be a member of the HTLV family and designated it HTLV-III. Here we show that certain sequences of the genome of HTLV-III and both HTLV-I and HTLV-II are homologous, with the most conserved sequences being located within the *gag-pol* region and less but detectable homology occurring in the *env* and *pX* region.

Virus particles were purified from supernatant fluids of HT cells, clone 9 (H9) infected with HTLV-III (HTLV-III_B) by centrifugation through a sucrose density gradient at equilibrium (12). HTLV-III_B was originally obtained from pooled supernatants of short-term lymphocyte cultures of AIDS patients. Virus particles were also purified from normal peripheral blood lymphocytes newly infected by

virus of a primary leukocyte culture from another AIDS patient (HTLV-III_Z) (17). The particles were lysed with sodium dodecyl sulfate (SDS), digested with proteinase K, and directly chromatographed on an oligo(dT) cellulose column. The resulting polyadenylate [poly(A)]-containing RNA was used as template to synthesize ³²P-labeled complementary DNA (cDNA) in the presence of oligo(dT) primers. The size of the resultant cDNA ranged from 0.1 to 10 kb (not shown). When these labeled cDNA's were hybridized to poly(A)-containing RNA purified from infected and uninfected H9 cells as well as other uninfected human cell lines, only the infected H9 cells contained homologous RNA sequences as evidenced by discrete RNA bands after Northern hybridization. Figure 1 shows that cDNA preparations from HTLV-III_B and HTLV-III_Z gave identical patterns, detecting RNA spe-

cies of about 9.0, 4.2, and 2.0 kb. These bands are similar in size to those corresponding to genomic size messenger RNA (mRNA) and spliced mRNA's of *env* and *pX* sequences previously observed in cells infected with HTLV-I (15), consistent with the anticipated relatedness of these viruses. Furthermore, viral mRNA bands of HTLV-II-infected cells were detected with an HTLV-III cDNA probe (Fig. 1b, lane 6) and again the sizes of the mRNA were like those with HTLV-I.

To determine directly the homology between HTLV-III and HTLV-I and HTLV-II, we hybridized HTLV-III cDNA to cloned genomes of HTLV-I and HTLV-II digested with specific restriction endonucleases. Complete genomes of a prototype HTLV-I (16), an HTLV-I variant called HTLV-Ib (16), and HTLV-II were digested with two restriction enzymes as indicated in the

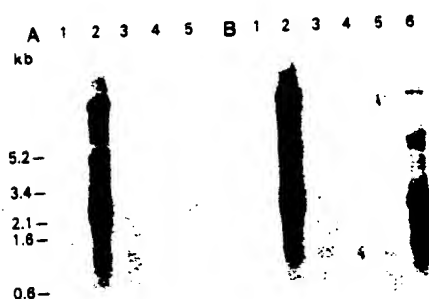


Fig. 1. HTLV-III-specific sequences in cellular RNA from HTLV-infected cells. Poly(A)-selected cellular RNA was size-separated by formaldehyde-agarose gel electrophoresis, transferred to Zeta probe membrane (Bio-Rad Labs) by electroelution and hybridized to (A) HTLV-III_B cDNA and (B) HTLV-III_Z cDNA. (A and B) Lane 1, uninfected H9 cells (5 µg); lane 2, HTLV-III_B-infected H9 cells (10 µg); lane 3, leukemic Jurkat cells (10 µg); lane 4, HTLV-I-infected C5/MJ cells (5 µg); and lane 5, HTLV-II-infected MO cells (5 µg). (B) Lane 6, a longer exposure of lane 5 in (B). Poly(A)-selected RNA was prepared by guanidine-HCl extraction and cesium chloride centrifugation followed by oligo(dT) cellulose chromatography as described (24). The cDNA was transcribed from poly(A)-selected virus-associated RNA with the use of oligo(dT) as a primer and avian myeloblastosis virus RNA-directed DNA polymerase as described (25). The hybridization was performed at 37°C for 16 hours in a mixture containing 40 percent formamide, 5× standard sodium chloride and sodium citrate (SSC: 0.15M NaCl and 0.015M sodium citrate, pH 7), 0.05M sodium phosphate buffer (pH 7), 5× PM (0.02 percent each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll 400), yeast RNA (200 µg/ml), denatured salmon sperm DNA (20 µg/ml), 0.1 percent SDS, and 10 percent dextran sulfate. The membrane was subsequently repeatedly washed with 2× SSC and 0.1 percent SDS at 62°C, air-dried, and exposed to a Kodak XAR film with the use of intensifying screens.



Fig. 2. Relatedness of the genome of HTLV-III_B with the genomes of HTLV-I and HTLV-II. Sites of digestion by the relevant restriction enzymes and the expected sizes of the fragments are shown below the gels. Cloned HTLV-I (λST), HTLV-Ib (λMC), and HTLV-II (pMO) are shown below the gels. Cloned HTLV-I (λST) DNA digested with Sst I plus Pst I and Sst I HTLV-III_BcDNA. Lanes 1 and 2, HTLV-I (λST) DNA digested with Sst I plus Pst I and plus Sal I, respectively; lanes 3 and 4, HTLV-Ib (λMC) DNA digested with Sst I plus Sal I, respectively; lanes 5 and 6, HTLV-II (pMO) DNA digested with Bam HI plus Sst I plus Sal I, respectively. HTLV-I (λST) and HTLV-Ib (λMC) clones were Xho I and Bam HI plus Eco RI, respectively. HTLV-I (λST) and HTLV-Ib (λMC) clones were obtained from the genomic libraries of DNA's from ATL patients S.T. and M.C., respectively. Both cellular DNA's were cloned at the Sst I site of phage λgtWES-λB DNA (16). HTLV-I (λST) is a prototype HTLV-I and HTLV-Ib (λMC) is a variant of HTLV-I that contains some divergent restriction enzyme sites, including the lack of the second Pst I site from the 5' end of the viral genome (16). HTLV-II (pMO) was obtained by subcloning λMO15A (26) at the Bam HI site of plasmid pBR322 DNA. The cDNA was synthesized as described in Fig. 1 and hybridization was performed at 37°C for 16 hours in a mixture containing 30 percent formamide, 5× SSC, 5× PM, denatured DNA (100 µg/ml), 0.1 percent SDS, and 10 percent dextran sulfate. The membrane was subsequently washed and exposed as described in Fig. 1.

legend to Fig. 2 and blot-hybridized to 32 P-labeled HTLV-III_B cDNA region spanning the *gag* and *pol* genes showed the greatest homology. For the prototype HTLV-I, this corresponds to the 1.7-kb Pst I-Pst I fragment and 5.3-kb Sst I-Sal I fragment. HTLV-Ib, which lacks a Pst I site indicated in parentheses in Fig. 2, revealed the expected 3.0-kb Pst I-Pst I fragment instead. Similarly, strong hybridization to the *gag-pol* sequences of HTLV-II also occurred. This is reflected in the 4.2-kb Bam HI-Xho I fragment and the 4.0-kb Bam HI-Eco RI fragment (Fig. 2, lanes 5 and 6).

Fragments corresponding to the *env* and *pX* sequences of HTLV-I and HTLV-II also hybridized weakly with HTLV-III_B cDNA (see the 2.4-kb Pst I-Pst I and the 2.1-kb Sst I-Pst I fragment in Fig. 2, lane 1) as did the 1.4-kb Pst I fragment of HTLV-Ib containing only *pX* sequences (Fig. 2, lane 4). The ease of detection of these sequences varied with different preparations of cDNA, probably because of variable representations of the 3' end of the virus genome. We used cDNA from both HTLV-III_B and HTLV-III_Z. Figure 3 shows the results for HTLV-III_Z cDNA. Subclones of HTLV-I containing different regions of the genome were hybridized to HTLV-III_Z cDNA (Fig. 3A). With the exception of fragment c, which corresponds to an internal portion of the *pol* gene, all fragments were detected by hybridization, including fragment a (LTR-*gag*) after long exposure of the autoradiogram. Similarly, the 3' half of HTLV-II contained in the 3.5-kb Bam HI-Bam HI fragment and the 2.3-kb Bam HI-Xho I fragment could be detected with this particular HTLV-III cDNA probe (Fig. 3B).

Retroviruses called LAV (or sometimes IDAV₁ and IDAV₂) have been isolated from patients with lymphadenopathy syndrome and AIDS (17). Although LAV has been reported to lack relatedness to HTLV-I and -II (17), further characterization of its proteins and nucleic acids may reveal that LAV is related to these viruses and is identical to or related to HTLV-III.

The present data showing that certain nucleotide sequences of HTLV-III are homologous to sequences of HTLV-I and HTLV-II support our proposal that this virus should be classified within the HTLV family. However, HTLV-III is much less related to HTLV-II and HTLV-I than HTLV-II and HTLV-I are to each other. It is of interest that still other HTLV-related T lymphotropic retroviruses have been identified in Old World monkeys (18). These primate vi-

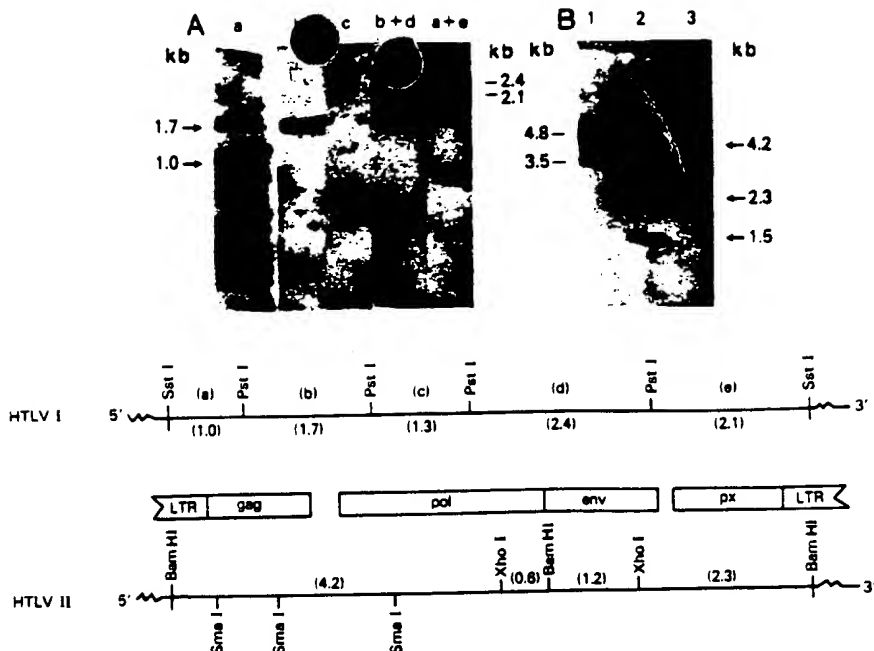


Fig. 3. Relatedness of the genome of HTLV-III_Z with the genomes of HTLV-I and HTLV-II. DNA from subclones of HTLV-I_{ST} and HTLV-II_{MO} was digested with the indicated restriction enzymes. Fragments were separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane (24), and hybridized with HTLV-III_Z cDNA. (A) HTLV-I subclones were constructed by "shotgun" cloning of fragments generated by codigestion with Pst I and Sst I into pBR322 containing fragments designated a to e on the illustrated restriction map of HTLV-I. The viral inserts were released by digestion with the appropriate enzymes. (B) HTLV-II (pMO) DNA: Lane 1, digested with Bam HI; lane 2, digested with Bam HI plus Sma I; lane 3, digested with Bam HI plus Xho I. The cDNA was synthesized as in Fig. 1 and hybridization was performed as in Fig. 2, except that the hybridization mixture contained 40 percent formamide.

ruses are closely related to HTLV-I and only minimally to HTLV-II (19). Although the most conserved sequences of HTLV-III are in the region spanning the junction of the predicted *gag* and *pol* genes, other weakly homologous sequences are also detected in the *env* and *pX* genes. Homology in the *gag* and *env* coding sequences has already been suggested by immunological cross-reactivity between these antigens derived from the three subgroups (14). Homology in the *pX* region is an additional demonstration that HTLV-III belongs to the HTLV family, which is unique among retroviruses in its possession of the *pX* genes (20, 21). It is interesting that *pX* is the most conserved region between HTLV-I and HTLV-II (21) and that both of these viruses can transform T cells in vitro. In contrast, the *pX* region is much less conserved in HTLV-III, a cytopathic virus that lacks transforming activity (11, 12).

Comparisons of the LTR regions between HTLV-I and HTLV-II have revealed a conserved 21-bp repeat sequence in two otherwise very divergent LTR's (22). The location of this sequence upstream of promoter sequences suggests that it is similar to other viral enhancer sequences. In view of the tro-

pism of HTLV-III for OKT4⁺ lymphocytes, it will be interesting to see if this virus also has such an enhancer sequence in its LTR. Our present study does not allow us to compare specifically the LTR of HTLV-III to those of HTLV-I and -II. However, the weak signal obtained with 5' and 3' ultimate fragments containing the LTR suggest that these elements have minimal or no homology.

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Growth Self-Incitement in Murine Melanoma B16:

A Phenomenological Model

Abstract. The growing murine melanoma B16 secretes increasing quantities of a substance or substances immunologically cross-reactive with insulin. The elevated concentrations of these substances in blood are accompanied by a decrease in blood glucose concentration and release of growth hormone, which is followed by increased tumor growth. By use of a phenomenological model based on these data, we show that B16 incites its own growth by positive feedback.

Certain human (1-5) and murine (6-8) tumors produce and secrete a substance or substances immunologically cross-reactive with insulin (SICRI's). Several features distinguish SICRI's from insulin and show that they are of tumor origin: (i) their high concentrations observed also in tumor-bearing diabetic patients (2, 5) and diabetic mice (6-9); (ii) the restoration of normal insulin concentrations after removal of the tumor (1, 2, 4); (iii) the high concentrations of SICRI's within tumor tissue (1, 2, 9, 10); and (iv) the lack of a correlation between concentrations of circulating SICRI's and C-peptide (5, 11). Yet SICRI's display insulin-like action in that they decrease blood glucose in tumor patients (2-5) and tumorous mice (6, 8, 9).

We now show that in murine melanoma B16 the concentration of SICRI's in blood is a function of tumor volume and that glucose concentration in blood is a function of SICRI concentration. The decreased amount of glucose in blood is correlated with elevated amounts of circulating growth hormone which, in turn, is paralleled by increased tumor growth. By use of a phenomenological model

based only on correlations of tumor volumes and SICRI and glucose concentrations in blood, we show that a positive feedback—that is, growth self-incitement—occurs in melanoma B16.

Male C57BL/H Irb mice 2.5 months of age and weighing 22 g each were housed five to a cage and given free access to water and standard pelleted food. The tumor, originally obtained from the Holt Radium Institute (Manchester, England), has been maintained at the Rugjer Bošković Institute since 1975 by subcutaneous inoculations of 2×10^6 cells into the flanks of recipient animals. Three opposite diameters (A, B, and C) of almost spherical prolate ellipsoid tumors were measured, and their volume was calculated as $V = ABC\pi/6$. Blood glucose concentrations were measured by the ortho-toluidine method (12). The SICRI concentrations were determined by insulin-specific radioimmunoassay (13) with the use of Phadebas kits (Uppsala, Sweden); therefore, these concentrations are relative and expressed as insulin equivalents (5).

Secreting tumors release SICRI's even in alloxan-diabetic mice (6). In normoin-

sulinemic animals with melanoma B16, SICRI concentrations in the blood may be more than five times greater than normal insulin concentrations and are correlated with tumor volume (Fig. 1A); SICRI's also appear in diabetic melanoma-bearing mice (9). By fractionating tumor extract on a Sepharose 6B column, we obtained an apparent relative molecular size for B16 SICRI of 120,000 (10), as in non-Hodgkin's lymphoma (5). Increase of tumor volume and of SICRI concentrations was accompanied by a decrease in blood glucose concentration (Fig. 1B). The correlation between amounts of SICRI and blood glucose was high.

Our phenomenological model is based on consideration of (i) exponential volume-SICRI and SICRI-glucose relations (Fig. 1) and (ii) the Gompertzian tumor growth model (14) modified to include positive feedback and chosen empirically because of its demonstrated applicability to tumor growth (14). Figure 2 shows the proposed feedback loop formulated by the following relations.

$$S = ae^{\alpha V}; a, \alpha > 0 \quad (1)$$

$$G = be^{-\beta S} = be^{-\alpha\beta e^{\alpha V}} = g(V); b, \beta > 0 \quad (2)$$

$$V = f(G, t) = V_0 e^{P(G)(t - t_0)}; V_0, \gamma > 0 \quad (3)$$

$$P_n(G) = a_1 + a_2 G + \dots + a_n G^{n-1} \quad (4)$$

where S and G denote SICRI and glucose concentrations, respectively, V_0 the initial tumor volume, and V the tumor volume at time (t) after transplantation of the tumor. The symbols a , b , α , β , γ , a_1 , a_2 , \dots , a_n are parameters obtained by the least-square fitting of the empirically chosen functions 1 to 3 to the data (see Table 1). The parameter a_2 differs significantly from zero, while a_3 (and also a_4 , a_5 , \dots , a_n) can with fair confidence be taken as zero according to the value of F [see (15)]. Thus, volume appears to depend significantly on glucose concentration; this dependence is described by a simple exponential function.

The feedback can be measured by calculating the open-loop gain parameter (Ω) [see (16)]. Here the infinitesimal changes of tumor volume (dV) and glucose concentration (dG) according to Eqs. 2 and 3 are

$$dV = \left. \frac{\partial f(x, t)}{\partial x} \right|_{x=G} dG + \left. \frac{\partial f(G, y)}{\partial y} \right|_{y=t} dt \quad (5)$$

$$dG = \left. \frac{\partial g(z)}{\partial z} \right|_{z=V} dV \quad (6)$$

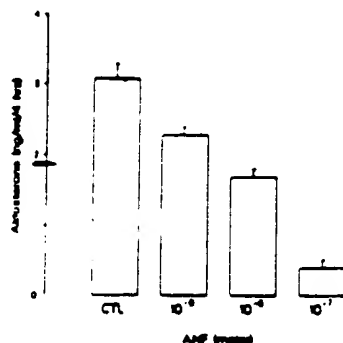


Fig. 1 Effect of ANF(8-33) on basal aldosterone secretion. Rat glomerulosa cells were prepared by enzymatic digestion of 20 rat adrenals after enucleation. The cells remaining on the capsule were digested for 30 min with a mixture of collagenase and DNase (4 mg ml^{-1} , 4 mg ml^{-1}) for 30 min. Dispersed cells were filtered through gauze and centrifuged at 800 r.p.m. for 15 min. The pellet was resuspended in M199 buffer containing 0.1% bovine serum albumin (BSA) and the cells centrifuged at 800 r.p.m. for 15 min. The cell pellet was again resuspended in M199-0.1% BSA buffer and distributed in 900- μl aliquots to 12×75 plastic tubes. The samples were preincubated for 90 min in a 37°C waterbath under an atmosphere of 5% $\text{CO}_2/95\%$ O_2 . Aliquots of the test samples were added in a 100 μl volume and incubated for 4 h. Aldosterone and corticosterone were measured by radioimmunoassay using antisera purchased from Endocrine Sciences, Oxnard, California, and ^3H -labelled steroid from NEN. Results are the mean \pm s.e.m. of seven replicates. Statistical analysis was performed by analysis of variance and all points are significant ($P < 0.01$) from control. (ANF(8-33) was the gift of Drs R. Hirschmann and D. F. Veber of Merck, Sharp and Dohme Research Laboratories).

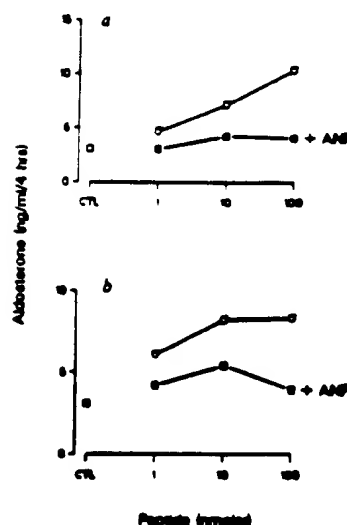


Fig. 2 Effect of ANF(8-33) on stimulated aldosterone secretion. **a**, Rat glomerulosa cells were prepared as described in Fig. 1 and incubated with synthetic human ACTH either alone (open circles) or in combination with equimolar amounts of ANF(8-33) (closed squares). Aldosterone secretion was measured as above by radioimmunoassay. **b**, Cells were incubated with synthetic angiotensin-II (AN-II) either alone (open circles) or in the presence of equimolar amounts of ANF(8-33) (closed squares). Control cells received neither peptide, thereby indicating the ability of ANF to decrease aldosterone production to basal levels. Results are the mean \pm s.e.m. of 7 replicates and all points are significant when compared with their respective control ($P < 0.001$). Synthetic hACTH(1-39) and angiotensin II were synthesized by Dr Nicholas Ling by solid-phase methodology.

(8-33) as a natriuretic hormone, and now in inhibiting basal and stimulated aldosterone formation, suggests that its biological activities are an integral part of the homeostatic mechanisms regulating sodium retention. Furthermore, unlike somatostatin, its inhibitory effect is not restricted to angiotensin-stimulated aldosterone secretion, but affects the formation of both basal and stimulated mineralocorticoids. Moreover, at no point was ANF(8-33) observed to stimulate aldosterone. The observations reported here provide the groundwork for defining the mechanisms by which atrial-derived peptides affect sodium retention and suggest that this peptide may be responsible for the attenuated effects of AN-II on the adrenal cortex during sodium loading^{16,17}. The understanding of some clinical forms of idiopathic hypo- and hypertension^{18,19} may therefore result from defining the interactions between ANF, the adrenal cortex and the basic mechanisms regulating ANF secretion.

After submission of this manuscript, Chartier *et al.*²⁰ and DeLean *et al.*²¹ reported findings similar to those reported here.

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Molecular cloning of lymphadenopathy-associated virus

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Lymphadenopathy-associated virus (LAV) is a human retrovirus first isolated from a homosexual patient with lymphadenopathy syndrome, frequently a prodrome or a benign form of acquired immune deficiency syndrome (AIDS)¹. Other LAV isolates have subsequently been recovered from patients with AIDS or pre-AIDS²⁻⁴ and all available data are consistent with the virus being the causative agent of AIDS. The virus is propagated on activated T lymphocytes and has a tropism for the T-cell subset OKT4 (ref.

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6), in which it induces a cytopathic effect. The major core protein of LAV is antigenically unrelated to other known retroviral antigens^{1,2,7}. LAV-like viruses have more recently been independently isolated from patients with AIDS and pre-AIDS. These viruses, called human T-cell leukaemia/lymphoma virus type III (HTLV-III)⁸⁻¹¹ and AIDS-associated retrovirus (ARV)¹², seem to have many characteristics in common with LAV and probably represent independent isolates of the LAV prototype. We have sought to characterize LAV by the molecular cloning of its genome. A cloned LAV complementary DNA was used to screen a library of recombinant phages constructed from the genomic DNA of LAV-infected T lymphocytes. Two families of clones were characterized which differ in a restriction site. The viral genome is longer than any other human retroviral genome (9.1–9.3 kilobases).

The cDNA first-strand of LAV was synthesized in an endogenous, detergent-activated reaction. LAV virions were purified from the supernatant of FR8 cells, a B-lymphoblastoid LAV-producing line¹³, and the reaction was primed with oligo(dT). Three cDNA clones, pLAV13, 75 and 82, carrying inserts of 2.3, 0.6 and 0.8 kilobases (kb), respectively, were characterized further (Fig. 1). All three inserts have a common restriction pattern at one end, indicative of a common priming site. The 50-base pair (bp) common *Hind*III–*Pst*I fragment was sequenced and shown to contain an oligo(dA) stretch preceding

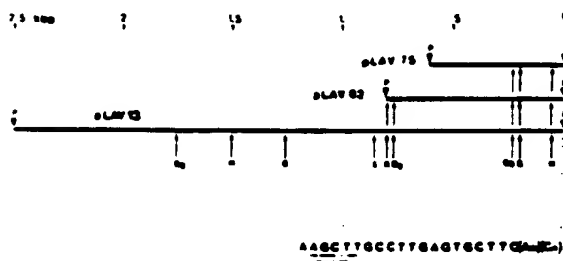


Fig. 1 Restriction maps of cDNA clones derived from LAV genomic RNA. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; S, *Sac*I; X, *Xho*I.

Methods: LAV cDNA was synthesized in an endogenous detergent-activated reaction. For each reaction, LAV virions were purified on a 20–60% sucrose gradient as described previously¹, from 200 ml of supernatant of the LAV-producing FR8 line¹³. Virus-containing fractions were pooled, diluted with NTE buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA) and centrifuged (Beckman type SW56 rotor, 50,000 r.p.m., 60 min). The viral pellet was resuspended in 250 µl of NTE. Reaction volume was adjusted to 1 ml and final concentrations were: 50 mM Tris-HCl pH 7.8, 25 mM NaCl, 6 mM MgCl₂, 10 mM dithiothreitol, 0.02% Triton X-100, 0.1 mM of each of dATP, dGTP, TTP, 4 µM dCTP including 200 µCi of [α -³²P]dCTP (400 Ci mmol⁻¹, Amersham) and 50 µg ml⁻¹ oligo(dT) primer. Incubation was at 37 °C. After 15 min, dCTP was added to 25 µM. At 45 min, the reaction was stopped with EDTA and SDS (final concentrations 20 mM and 0.5%, respectively). After 1 h of proteinase K digestion (100 µg ml⁻¹, 37 °C), the reaction mixture was extracted with phenol/chloroform and cDNA-RNA hybrids were ethanol-precipitated. Second-strand synthesis with enzyme-free DNA polymerase I (Boehringer) and RNase H (BRL) and dC-tailing with terminal transferase (Boehringer) were performed according to Gubler and Hoffman¹⁴. Tailed double-stranded cDNA was annealed to dC-tailed *Pst*I-linearized pBR327 vector. *Escherichia coli* C600 recBC was transformed by the CaCl₂ method; 500 recombinant clones were screened in situ¹⁵ with a ³²P-labelled LAV cDNA in which the first strand had been synthesized as described above, except that an alkaline hydrolysis step was included. Approximately 10% of recombinants proved positive, the majority of which formed a family of cross-hybridizing clones. Three recombinants, pLAV13, pLAV75 and pLAV82, carrying inserts of 2.3, 0.6 and 0.8 kb, respectively, were analysed further. There are no sites for *Eco*RI, *Nru*I, *Pvu*I, *Sac*I, *Sma*I, *Sna*I or *Xba*I in the pLAV13 insert. The *Hind*III–*Pst*I fragment was subcloned into M13mp8 and sequenced according to Sanger *et al.*¹⁶ using a 15-mer primer (Biolabs) and [α -³²P]dCTP (Amersham).

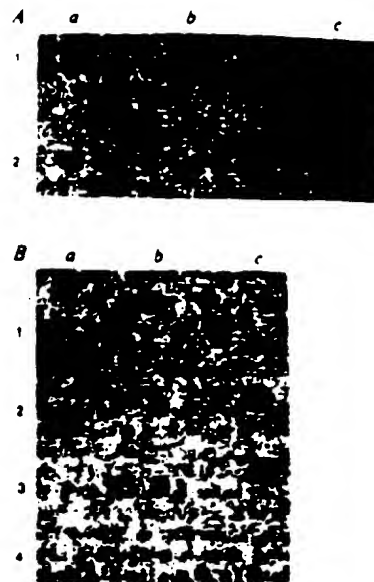


Fig. 2 Rapid dot-blot technique for LAV detection in cell culture supernatant. Spots represent: A, a, 1 µl; b, 2 µl; c, 4 µl of concentrated (250×) cell culture supernatant from (1) LAV-producing CEM cells (reverse transcriptase activity (RT), determined as described previously¹, was 140,000 c.p.m. ml⁻¹); (2) LAV-producing Epstein-Barr-transformed B-cell line FR8 (RT 175,000 c.p.m. ml⁻¹); B, a, 1 µl; b, 2 µl; c, 5 µl of 100× concentrated supernatant from (1) uninfected normal T lymphocytes (no RT activity); (2) LAV-producing normal T lymphocytes (RT 170,000 c.p.m.); (3) LAV-producing CEM line (RT 150,000 c.p.m.); and (4) culture of bone marrow lymphocytes from a haemophilic patient⁴ with AIDS (RT 7,000 c.p.m.). **Methods:** Cell culture supernatants were pelleted through 0.5 ml 20% sucrose cushions in NTE buffer (Beckman type SW56 rotor, 50,000 r.p.m., 1 h, 4 °C). The pellet was resuspended in NTE buffer as indicated. Concentrated virus was spotted onto dried nylon filters (Zetabind) presoaked in 20× SSC (3 M NaCl, 0.3 M sodium citrate). After baking (at least 30 min at 80 °C), filters were hybridized with ³²P nick-translated pLAV13 insert (Fig. 1) (specific activity >10⁶ c.p.m. per µg) for 12–16 h in stringent conditions (50% formamide, 5× SSC, 42 °C), washed (0.1× SSC, 0.1% SDS, 65 °C, 2×30 min), and exposed for 20 h (Kodak XAR5 film with an intensifying screen) at –70 °C.

the cloning dC tail. The clones are thus copies of the 3' end of a poly(A) RNA.

The specificity of pLAV13 was determined in a series of filter hybridization experiments using nick-translated pLAV13 insert as a probe. First, using an adapted spot-blot technique, we could detect LAV virion RNA from normal T cells, FR8 and other B-cell lines and CEM cells (L.M. and R. Weiss, unpublished results; Fig. 2). LAV was also detected in a bone marrow cell culture (Fig. 2B, line 4) from a haemophilic with AIDS⁴, in spite of the low titre of virus in the supernatant. Uninfected cultures proved negative (Fig. 2B, line 1). Second, the probe detected DNA in the Southern blots of LAV-infected T lymphocytes and CEM cells (Fig. 3). No hybridization was detected in DNA from uninfected lymphocytes or from normal liver (data not shown) in the same hybridization conditions. A characteristic 1.45-kb *Hind*III fragment which co-migrated with an internal viral fragment in *Hind*III-cleaved pLAV13 (Fig. 1) was detected in the Southern blots. Bands at 2.3 and 6.7 kb were also detected. Together, these data show that pLAV13 DNA is exogenous to the human genome and detects both RNA and integrated DNA forms derived from LAV-infected cells. Thus, pLAV13 is LAV specific. Being oligo(dT)-primed, pLAV13 must contain the R and U3 regions of the long terminal repeat (LTR) as well as

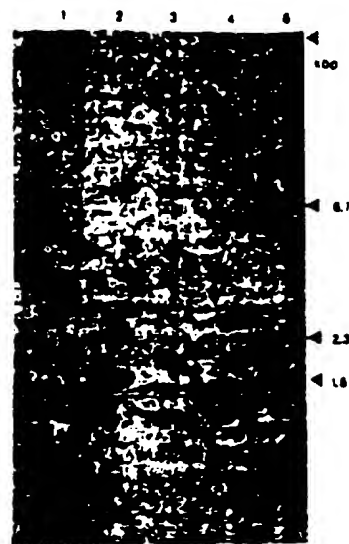


Fig. 3 Southern blot of *Hind*III-restricted genomic DNA from LAV-infected and uninfected cells hybridized with pLAV13. *Hind*III-restricted high relative molecular mass DNA from: lane 1, uninfected CEM cells; lane 2, LAV-infected CEM cells; lane 3, uninfected T cells after 5 days' culture; lane 4, LAV-infected T cells 2 days after infection; lane 5, LAV-infected T cells 5 days after infection.

Methods: Peripheral blood T lymphocytes of a healthy donor were stimulated for 3 days with phytohemagglutinin, after which they were infected with LAV (isolate BRU-LAVI) at 10^6 c.p.m. reverse transcriptase activity per 10^6 cells as described previously¹, except for part of the culture kept uninfected for controls. Two and five days after infection, genomic DNA was extracted. *Hind*III-digested DNA (10 μ g) was electrophoresed through a 0.8% agarose gel and Southern blotted. The filter was hybridized in 10 ml of 50% formamide, 5 \times SSC, 1 \times Denhardt's, 10% dextran sulphate with 100 μ g ml⁻¹ denatured sonicated salmon sperm DNA and 2 $\times 10^7$ c.p.m. of nick-translated pLAV13 insert (4×10^6 c.p.m. per μ g) for 10 h at 42 °C. The filter was washed at 68 °C in 0.1 \times SSC, 0.1% SDS for 2 \times 30 min and exposed to Kodak XAR5 film at -70 °C for 16 h using an intensifying screen.

the 3' end of the coding region, assuming a conventional retroviral genome structure.

Having found a *Hind*III site about 20 bp 5' of the poly(A) stretch and thus within the R region of the LTR, we cloned the LAV genome by making a partial *Hind*III digest of genomic DNA from LAV-infected T cells of a healthy donor. A 9 \pm 1.5-kb DNA-containing fraction was precipitated and ligated into the *Hind*III arms of phage vector λ L47.1 (ref. 14). When nick-translated pLAV13 insert was used as a probe to screen $\sim 2 \times 10^6$ phage plaques *in situ*, five independent clones were obtained. A restriction map of clone λ J19 and of a *Hind*III variant, λ J81, are shown in Fig. 4. Recombinants λ J27, λ J31 and λ J57 have the same *Hind*III map as λ J19, while λ J81 is so far unique. As the two clones were derived from the first isolate¹ of LAV reported (isolate BRU, or LAVI), we refer to the two viral genomes as LAV1a (λ J19) and LAV1b (λ J81). λ J19 shows four *Hind*III bands of 6.7, 1.45, 0.6 and 0.52 kb, the first two of which correspond to bands in the genomic blot of *Hind*III-restricted DNA (Fig. 3, lane 5). The smallest bands (0.6 and 0.52 kb) were not seen in the genomic blot, but the fact that they appear in all the independently derived clones analysed indicates that they represent internal and not junction fragments, assuming random integration of LAV proviral DNA. However, the 0.52-kb band hybridizes with pLAV13 DNA (Fig. 4) through the small *Hind*III-*Pst*I fragment of pLAV13. Thus, the 0.5-kb *Hind*III fragment of λ J19 contains the R/US junction within the LTR. The finding of two small *Hind*III fragments in the 5' region reinforces

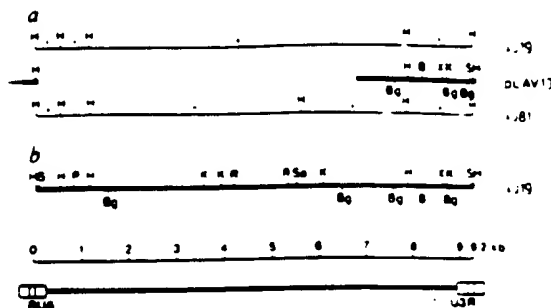


Fig. 4 Restriction maps of LAV proviral DNA in clones λ J19 (LAV1a) and λ J81 (LAV1b). a, *Hind*III restriction maps of LAV proviral DNA in clones λ J19 and λ J81. Those *Hind*III fragments detected by pLAV13 are marked by +, those not, by -. The restriction map of the pLAV13 cDNA clone is also shown. b, Restriction map of λ J19. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Sa, *Sal*I; X, *Xba*I. Beneath the scale is a schema for the general structure of retroviruses showing the LTR elements U3, R and U5. Only the R/U5 boundary has been defined (Fig. 1) and other boundaries are drawn only figuratively.

Methods: DNA from LAV-infected T cells was partially digested with *Hind*III and fractionated on a 5–40% sucrose gradient in 10 mM Tris-HCl pH 8, 10 mM EDTA, 1 M NaCl (Beckman type SW41 rotor, 16 h, 40,000 r.p.m.). A single fraction (9 ± 1.5 kb) was precipitated with 20 μ g ml⁻¹ dextran T40 as carrier and taken up in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). λ L47.1 (ref. 14) *Hind*III arms were prepared by first ligating the *cos* sites followed by *Hind*III digestion and fractionation through a 5–40% sucrose gradient as above. Fractions containing only the *Hind*III arms were pooled, precipitated and taken up in TE buffer. Ligation of arms to DNA was made at ~ 200 μ g ml⁻¹ DNA using a 3:1 molar excess of arms and 300 U of T4 DNA ligase (Boehringer). *In vitro* packaging lysates were made according to ref. 29. After *in vitro* packaging, the phage lysate was plated out on NM538 or a C600 recBC strain. Approximately 2×10^6 plaques were screened by *in situ* hybridization³⁰ using nitrocellulose filters. Hybridization was performed at 68 °C in 1 \times Denhardt's solution, 0.5% SDS, 2 \times SSC, 2 mM EDTA. Probe: ³²P nick-translated insert of pLAV13 at $>10^7$ c.p.m. per μ g. Filters were washed for 2 \times 30 min in 0.1 \times SSC 10.1% SDS at 68 °C, and exposed to Kodak XAR-5 film for 24–40 h with intensifying screens at -70 °C. Seven positive clones were identified and plaque-purified on a C600 recBC strain. Liquid cultures were grown and the recombinant phages banded in CsCl. Phage DNA was extracted and digested in the appropriate conditions. The restriction maps were orientated by hybridizing blots to pLAV13 DNA, which maps the 3' coding sequences of the viral genome as well as the U3-R region of the LTR. All cloning and amplification of LAV genomic clones was carried out in a P3 laboratory.

the usefulness of cloning LAV by partial restriction of genomic DNA.

λ J81 seems to be a restriction site polymorph of λ J19, showing five *Hind*III bands of 4.3, 2.3, 1.45, 0.6 and 0.52 kb (Fig. 4). The 2.3-kb band is readily detected in the genomic blot by a pLAV13 probe, although the 4.3-kb fragment is not. The finding that nick-translated λ J19 DNA hybridizes to all five *Hind*III bands of λ J81 in stringent hybridization and washing conditions indicates that λ J81 is a *Hind*III variant and not a recombinant virus. Also, other mapped restriction sites in λ J81 are identical to those of λ J19 (not shown). Thus, the *Hind*III restriction pattern in the Southern blot can be explained by variation within the single isolate of LAV used to infect the T cells.

HTLV-II¹³ and HTLV-II¹⁶ constitute a pair of C-type transforming retroviruses with a tropism for the T-cell subset. OKT4 Both genomes (comprising one LTR) are ~ 8.3 kb long^{17,18}, have an X region and show extensive sequence homology. They hybridize between themselves in reasonably stringent conditions (40% formamide, 5 \times SSC) and the X regions hybridize even at 60% formamide¹⁹. Thus, a conserved X region is a hallmark of

this class of virus. We have compared cloned LAV DNA and cloned HTLV-II DNA (pMO)²⁰ by blot-hybridization and find no cross-hybridization in low stringency conditions of hybridization and washing ($T_m = 55^\circ\text{C}$), even after 2 days' exposure at -70°C using intensifying screens (data not shown).

The human T-lymphotropic retroviruses HTLV-III⁸ and ARV¹², recently isolated from patients with AIDS or pre-AIDS, have similar morphological, biochemical and immunological properties to LAV, which suggests that they probably represent different isolates of the LAV prototype. DNA hybridization between HTLV-III and HTLV-I and -II has been reported, most noticeably at the gag-pol junction and less so in the characteristic X region of HTLV-I and -II¹¹. As mentioned above, we could detect no such hybridization and conclude that the reported homology must have been due to either (1) the use of an uncloned cDNA as hybridization probe, (2) the fact that the isolates in question differ substantially from those we have cloned, or (3) the possibility that HTLV-III and a HTLV-I/II-like virus were co-infecting the cells. The last possibility may also apply to the preliminary report of cross-hybridization between a LAV-like virus and a cloned HTLV-II DNA probe⁷. Thus, we find no molecular evidence of a relationship between LAV and HTLV. Furthermore, the LAV genome is ~ 9 kb long, compared with 8.3 kb for the HTLV viruses^{17,18}. Despite their comparable genome sizes, LAV does not cross-hybridize with Visna virus²² (~ 9 kb) (data not shown) or with several human endogenous viral genomes (ref. 23 and M. Martin, personal communication) in non-stringent conditions ($T_m = 55^\circ\text{C}$). These data and morphological and immunological dissimilarities^{1,2} between LAV and the HTLV-I/-II pair all point to LAV being a novel class of human retrovirus.

In conclusion, we have molecularly cloned the complete genome of LAV from freshly infected activated T cells of a healthy donor. It has been shown that the tropism of certain retroviruses resides in the LTR^{24,25} and that sequence differences and insertions/deletions are present in the LTRs of leukaemogenic and non-leukaemogenic retroviruses. It is thus possible that LAV and LAV-like viruses passaged through B- and T-transformed cell lines^{9,12,13} might have undergone some attenuation. Although the cDNA clones were made from a LAV-producing B-cell line, the genomic clones were isolated from LAV-infected normal T cells. Thus, the clones represent LAV genomes that have not been selected or adapted to a particular cell line. However, the LAV genome is shown to be polymorphic even within a single isolate and independent isolates will probably differ widely.

The availability of cloned LAV DNA should facilitate the understanding of the molecular mechanism of viral replication, and the tropism of the virus. The DNA sequence of LAV opens up the possibility of expressing the viral gag and env gene products and of studying the molecular basis of LAV antigenicity.

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Molecular cloning of AIDS-associated retrovirus

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Retroviruses cause a wide variety of diseases in avian and mammalian species. Human acquired immune deficiency syndrome (AIDS) leads to collapse of the immune system and death by a wide variety of opportunistic infections; several forms of cancer are associated with this syndrome. Retroviruses have been recovered from tissues of AIDS patients and from patients with related conditions. These similar slowly-isolated viruses are lymphadenopathy-associated virus (LAV)¹, human T-cell lymphotropic virus (HTLV-III)^{2,3} and AIDS-associated retrovirus (ARV-2)⁴. We have identified a RNA genome of ~ 9 kilobases (kb) in viruses purified from the culture medium of a human T-cell tumour line infected with ARV-2. A cDNA probe made from viral RNA detected circular DNA molecules and proviral forms in infected cells. We prepared a library of infected cell DNA. Recombinant phage included those with a 9.5-kb proviral DNA and viral DNA permuted with respect to the single *EcoRI* site. Comparison of three ARV isolates from different AIDS patients revealed polymorphism of restriction endonuclease sites.

HUT-78 cells, originating from a human T-cell lymphoid tumour⁵, were used to propagate the ARV-2 strain of virus⁴. To characterize the viral genome, RNA was extracted from purified virions and electrophoresed on agarose gels containing methyl mercury hydroxide⁶. A distinct ~ 9 -kb RNA species was observed (Fig. 1) with smaller heterogeneous RNA and some ribosomal RNA species. The 9-kb RNA species was used as a template with random primers in a reverse transcriptase reaction to produce a virus-specific cDNA probe⁷. RNA of virus obtained from cells infected with ARV-2 or with two additional isolates, ARV-3 and ARV-4, showed distinct bands at 9 kb that hybridized with the cDNA probe (Fig. 1).

With this cDNA probe, we examined the structure of viral DNA in infected cells by digestion with restriction enzymes, electrophoresis in agarose gels and Southern blotting. No specific bands were detected in several digests of DNA from uninfected cells (Fig. 2a, lanes C, E), whereas bands were seen in infected cells (Fig. 2a, lane A). Undigested DNA from infected cells contained a species at 5.5 kb, a faint species at 9 kb



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Micrographs of an icosahedral 'flower' obtained by solidification of an Al-Li-Cu alloy were generated by Professor Guinier on an image processor starting from a scanning electron micrograph and using pseudo-colours. See News and Views p.640.

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Genome organization and transactivation of the human immunodeficiency virus type 2

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Analysis of the nucleotide sequence of the human retrovirus associated with AIDS in West Africa, HIV-2, shows that it is evolutionarily distant from the previously characterized HIV-1. We suggest that these viruses existed long before the current AIDS epidemics. Their biological properties are conserved in spite of limited sequence homology; this may help the determination of the structure-function relationships of the different viral elements.

THE acquired immune deficiency syndrome (AIDS) has now spread worldwide and appears to be an acute public health problem in Africa in particular¹⁻⁵. A retrovirus designated human immunodeficiency virus (HIV), but previously known as LAV, HTLV-III or ARV, was shown to cause AIDS in the different areas afflicted by the epidemics⁶⁻⁸. Indeed, isolates from North America, Western Europe and Central Africa have the same biological properties, and antigenically cross-reactive proteins with the same relative molecular mass⁹⁻¹¹. Only studies at the molecular level have revealed some differences in the nucleotide sequence of North-American and African isolates^{12,13}. This sequence variation is also present, though to a lesser extent, among different isolates from the USA¹⁴⁻¹⁸.

The western part of Africa seemed relatively spared by AIDS³. Recently, however, several typical cases were found in a survey of patients from Guinea Bissau and other countries of West Africa¹⁹⁻²¹. Unexpectedly, most of these patients did not have detectable titres of antibodies against HIV. But they were found to be infected by a retrovirus related to HIV by its ultrastructural and biological properties, such as cytopathogenicity and tropism for cells carrying the CD4(T4) antigen¹⁹. Antibodies raised against HIV could immunoprecipitate the *gag* and *pol* products of these isolates, which have molecular masses that are similar but not identical to these antigens of HIV; in contrast, the *env* products could not be immunoprecipitated, whereas previous HIV isolates showed wide cross-antigenicity of the envelope glycoprotein. Furthermore, the genome of this new retrovirus cross-hybridized only poorly in very low stringency conditions with HIV DNA probes^{19,22}. We have therefore designated this West African AIDS virus as HIV type 2 (HIV-1 referring to the AIDS retrovirus previously identified in Central Africa, North America and Europe). More than 20 isolates have so far been made from patients with AIDS and related conditions, mainly originating from west Africa^{20,21}, but also in some Europeans (L.M., unpublished), and epidemiological studies in progress indicate a seroprevalence of 1-2% in some populations of West Africa (F. Brun-Vézinet, personal communication).

HIV-2 appears to be closely related to the simian immunodeficiency viruses (SIV) a group of cytopathic retroviruses whose prototype, STLV-3_{mac}, was identified in captive rhesus monkeys (*Macaca mulatta*) with an AIDS-like disease²³, and was later found to infect other primate species, either wild or in captivity²⁴⁻²⁶. Genetic comparisons of SIV, HIV-1 and HIV-2 may help to elucidate the phylogeny of these viruses and the origins of the recent AIDS epidemics. As these retroviruses share most of their biological properties, the identification of conserved

sequences is important to localize the functional domains of the viral proteins and regulating elements, and design new diagnostic and therapeutic tools. We present here the complete nucleotide sequence of HIV-2, the comparison of its proteins with those of HIV-1, and preliminary studies on the regulation of HIV-2 expression.

Nucleotide sequence and LTR analysis

The sequence presented in Fig. 2 is derived from two λ clones corresponding to integrated proviral DNA from the ROD isolate of HIV-2 (ref. 22), obtained in 1985 from an AIDS patient from Cape Verde Islands (offshore Senegal, refs 19, 20). The genome of HIV-2 is 9,671 nucleotides long (in its RNA form), whereas HIV-1 isolates are about 9,200 nucleotides long. This difference is partly explained by the respective sizes of the long terminal repeats (LTRs, see below).

The genetic organization of HIV-2 (shown in Fig. 1) is analogous to that of HIV-1, that is:

5'LTR-*gag-pol*-central region-*env*-orf F-3'LTR.

The 'central region', also identified in the ovine lentivirus visna²⁷, contains five major open reading frames (ORFs), four being clearly related to the ORFs of HIV-1 that encode the Q (or *sor*), R, *tat* and *art* (or *trs*) genes of HIV-1 (refs 15-18, 27-31). The fifth, which we designate ORF X, has no obvious counterpart in HIV-1. Alignments of the nucleotide sequences of HIV-1 and 2 show their distant homology (from ~60% for the more conserved *gag* and *pol* genes, to 30-40% for the other viral genes and LTRs). To allow these alignments to be made many insertions and deletions must be introduced into the sequences. We do not find that these insertions are the small duplications that would be characteristic of the recent divergence of retroviral sequences, as was noted among isolates of HIV-1 (ref. 12).

The limits of the LTRs and of their internal U3, R and U5 elements, determined by sequence analysis and some complementary experiments, are shown in Fig. 2. Classically bounding the retroviral LTRs are short inverted repeats (5' CTG-CAG 3') located after a polypurine tract for the 3' LTR, and before a sequence complementary to the 3' end of a transfer RNA that is used as primer by the reverse transcriptase (here, as in HIV-1 and visna virus, a lysine tRNA, refs 15, 27) for the 5' LTR. The R-U5 junction, corresponding to the 3' end of the polyadenylated viral RNA, was previously localized by sequencing oligo(dT)-primed complementary DNA (cDNA) derived from the HIV-2_{ROD} genome²². The length of U5 + R, and hence the position of the U3-R junction corresponding to the 5' cap site of the viral RNA were deduced from the size of a HIV-2 cDNA synthesized using the endogenous reverse transcriptase activity and the endogenous tRNA^{lys} primer (see Fig. 3). This

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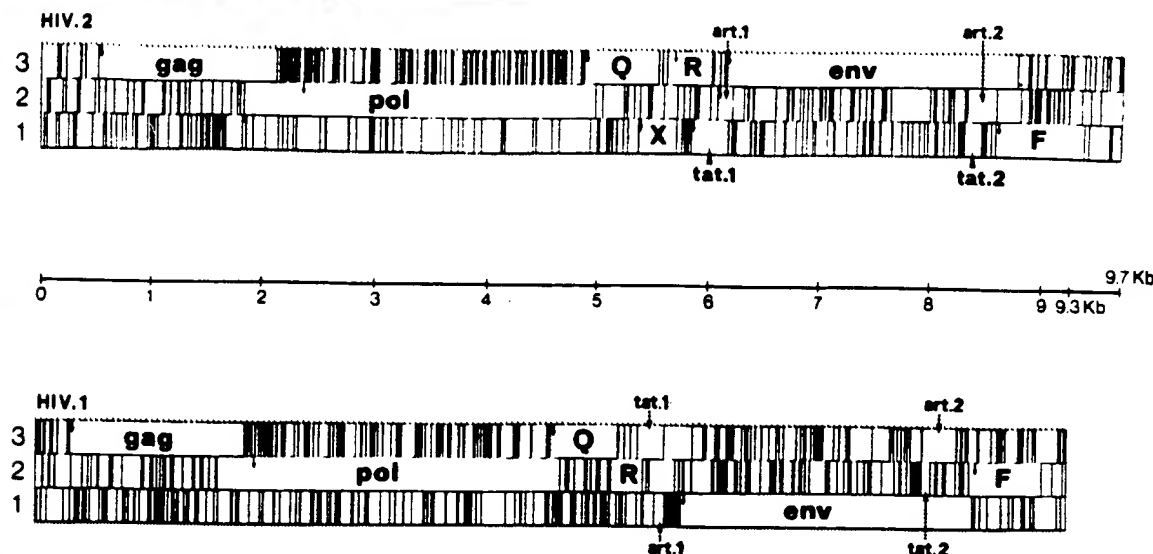


Fig. 1 Organization of the HIV-2 and HIV-1 genome (BRU isolate, ref. 15). Vertical bars represent the stop codons in the 3 reading frames. Arrows indicate the initiator AUG codons in viral genes or potential genes. *Tat* 1 and 2, *art* 1 and 2 are the open reading frames containing the coding exons of the *tat* and *art* genes.

strong-stop cDNA' is 302 \pm 1 nucleotides long (181 nucleotides in HIV-1, ref. 15). Thus, the U5 element is 125 bp long, U3 is 556 bp and R 173 bp (respectively 82, 456 and 97 bp in HIV-1). All the elements of the HIV-2 LTRs are larger than in HIV-1, and alignment by computer programs shows large insertions and very distant overall homology for the aligned regions²². However, the three Sp1 binding sites identified in HIV-1 (ref. 32), are also present in HIV-2 from nucleotide 9,419 to 9,448 with 17 out of 29 nucleotides homologous to this region of HIV-1. The core enhancers identified in HIV-1 (ref. 33) are present in HIV-2 from nucleotide 9,389 to 9,416: the first is 50% homologous and the second 100% homologous to that in HIV-1 (Fig. 2).

The analysis of the virus-specific poly(A)⁺ RNA (not shown) from a cell line infected with and continuously producing HIV-2 revealed a pattern of transcription reminiscent of that observed in HIV-1-infected cells: RNA of over 9 kilobases (kb), corresponding to a full-length transcript, and three types of spliced messenger RNA of 5, 4.5 and 2 kb, also observed in HIV-1 (refs 18, 34).

The *gag* and *pol* proteins and HIV phylogeny

The *gag* precursor of HIV-2 has a calculated relative molecular mass of 57,100 (M_r 57.1K), consistent with the p55 antigen²⁰ seen by immunoprecipitation with patient sera, and is probably processed, by analogy with HIV-1, into the proteins designated p16, p26 and p12 (refs 19, 20). By analogy with the p18^{gag} of HIV-1, p16 would be at the amino terminus of *gag* and precede p26, whose amino terminus has been sequenced (H. Marquardt, personal communication) and starts with the proline residue at position 951. The carboxy-terminal part of the *gag* precursor encodes a p12 that contains the cysteine-rich consensus of the retroviral nucleic-acid-binding proteins also found twice in the p13^{gag} of HIV-1 (ref. 15). The HIV-2 *pol* ORF could encode the p64 and p36 antigens of HIV-2 (ref. 20) which by analogy correspond to the p68 and p34 (reverse transcriptase and endonuclease, respectively³⁵) of HIV-1.

The *gag* and *pol* proteins of HIV-1 and 2 were expected to share large conserved domains, as these HIV-2 proteins can be precipitated by antibodies in sera from patients infected with HIV-1. However, we found that only 58% and 59.4% of the amino acids of *gag* and *pol* respectively are identical to the

corresponding HIV-1 products (Table 1a), whereas the more distant isolates of HIV-1 (Zairian and US) show 90 to 95% amino-acid identity in these proteins (Table 1b and ref. 12). Several insertions and deletions have to be introduced in the alignments (data not shown), whereas they are rare in the comparisons of *gag* and *pol* genes between HIV-1 isolates. The *gag* and *pol* proteins of HIV-2 are no closer to those of the Zairian isolates than to the prototype HIV-1 (BRU isolate) isolated in 1983 from a French patient⁶ probably infected in the USA. Overall, the difference in *gag* and *pol* between HIV-1 and HIV-2 is of the same order as that observed among the group of the human T-cell leukaemia viruses (HTLV-I and II) and bovine leukaemia virus (BLV). However, this latter group displays a higher conservation in the envelope, 70% amino-acid identity between HTLV-I and HTLV-II, versus about 42% between HIV-1 and HIV-2 (see below). Alignments of different retroviral *pol* proteins (Table 1b) confirm that the HIVs form a subgroup that is more related to the lentiviruses visna and equine infectious anaemia virus (EIAV) than to any other human or animal retrovirus.

Homologous domains in *env*

The envelope glycoproteins of retroviruses are translated from a subgenomic viral mRNA (here probably the transcript of 4.5 kb). Addition of sugar residues (*N*-linked glycosylation) gives rise to a high- M_r precursor which is processed by proteolytic cleavage. The length of the leader sequence of the HIV-2 glycoprotein cannot be precisely determined by alignment with that of HIV-1 (experimentally found to be 32 amino acids long³⁶) because of a lack of sequence homology (Fig. 4). But the amino terminus of *env* contains a relatively hydrophobic stretch in the calculated hydropathy plot (not shown) that is probably the signal peptide. The potential cleavage site between the external envelope glycoprotein (120K) and the transmembrane protein (previously thought to be the 36K antigen¹⁹, and now putatively identified as a 40K antigen²⁰) is found at amino acid 505 (Fig. 4) immediately after the Lys-Glu-Lys-Arg sequence. This cleavage site aligns partly to one (Lys-Ala-Lys-Arg) of the two potential cleavage sites found in HIV-1 (the other being located after the Arg-Glu-Lys-Arg stretch). The calculated M_r of the extracellular glycoprotein (EGP) and of the transmembrane protein (TMP) of HIV-2 would be 57K and 41.7K respectively; the discrepancy

Fig. 2 Complete nucleotide sequence of the HIV-2_{ROD} provirus genome. The sequence of the 9,671 nucleotide coding strand from the cap site to the polyadenylation site of the RNA is shown together with the deduced amino-acid sequence of the viral proteins. The 3-3-bp inverted repeats bounding the LTR are underlined with arrows. The primer binding site (PBS) complementary to the 3' end of tRNA^{phe}, and polypurine tract (PPT) are underlined. The repeat of the PPT in the *pol* gene is also underlined. The limits of the U3, R and U5 elements of the LTR are indicated by arrows. The promoter (TATAAA), the three potential Sp1 factor binding sites (indicated by dotted lines) and the two core enhancer sequences (E, indicated by dashed lines) of U3 are shown; in R the polyadenylation signal AATAAA is underlined. The viral genes and potential genes are translated from the first AUG of the open reading frame (ORF), except *pol* which is translated from the beginning of the ORF. The end of translation is indicated by a filled circle. The amino terminus of the major core protein, the p26^{gag} was determined by a filled circle. SD at position 6,140 and SA at position 8,307 indicate the probable splice donor and acceptor sites of the intron separating the two coding exons of the *tat* and *ari* genes.

Methods. The sequence was determined by the M13 shotgun cloning and dideoxynucleotide chain terminator method^{37,38} as described²⁷ starting from the inserts of the λ phages ROD 27 and ROD 35 containing integrated proviral DNA²². ROD 27 corresponds to the 5' part of the genome, to the *EcoRI* site at position 2,658, whereas ROD 35 corresponds to the part of the genome 3' to this site. An in-frame TAG stop codon was found in ROD 35 *env* gene. The sequencing of the corresponding region in an oligo(dT)-primed HIV-2 cDNA clone E2, described in ref. 22) revealed that it was due to a C-to-T mutation at position 8,304.

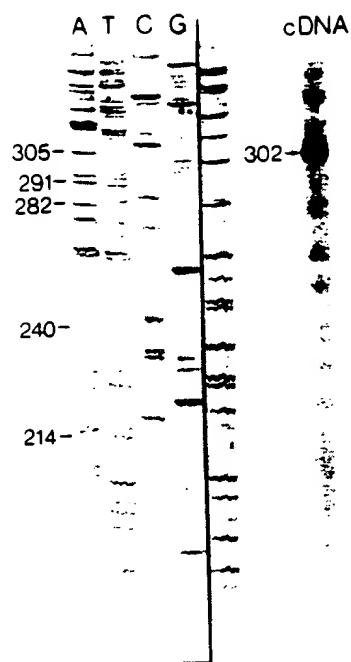


Fig. 3 HIV-2 strong-stop cDNA corresponding to the length of the R+U5 elements of the HIV-2 LTR. The methods were previously described¹⁵. Briefly, virions were purified by ultracentrifugation and an endogenous cDNA reaction performed with radiolabelled nucleotides after mild disruption of viral envelope with Triton X-100. The tRNA^{pst} primer, complementary to the PBS site flanking the U5 element at the 5' end of the genome, was then degraded by alkaline hydrolysis and the cDNA run on a denaturing 6% acrylamide-urea gel together with a sequence reaction for accurate estimation of the size of the products.

with the apparent M_r of the EGP is explained by glycosylation (30 sites in HIV-2, about half of which are conserved with respect to HIV-1).

Figure 4 shows an alignment of the envelopes of the two HIVs. The proteins are overall very distantly related (41.7% identity in the entire envelope, 39.4% in the EGP, 44.8% in the TMP) compared to divergent isolates of HIV-1 (about 75–80% identity in the whole envelope, ref. 12). Many large insertions have to be introduced, particularly in alignment of the EGPs where only short, widely separated domains are conserved between HIV-1 and 2. These domains are clustered into the conserved regions of the EGP of HIV-1 (identified by comparison of different isolates^{12–14}), and generally coincide with cysteine residues. Among the HIV-1 isolates, all the cysteine residues could be aligned in spite of the generally large genetic variation, especially in gp110. Almost all (22/23) of the cysteine residues of HIV-1 can also be aligned with HIV-2, but the latter contains seven additional cysteine residues, often in the regions representing insertions relative to HIV-1. Thus, the folding of the HIV-2 EGP could be different from that of HIV-1, and some regions, therefore, might be exposed in a different manner.

Other viral proteins

The HIV-1 genome contains several other genes encoding proteins of small M_r (10 to 27K), two of which (*tat* and *art*/*trs*) have an identified function: the positive regulation of viral expression^{30–33}. No role has yet been identified for the p23 encoded by ORF Q (or *src*)^{37,38}, nor for the p27 encoded by ORF F (or 3' ORF)³⁹. We also observed in the region between the *pol* and *env* genes of HIV-1 (central region) another potential gene, which we designated R (ref. 12). All these elements are found in HIV-2, but the corresponding proteins are only distantly homologous (see Table 1a). In the F protein, most of the difference between HIV-1 and 2 is due to a large insertion in

the amino terminus of HIV-2. The second half of the protein, encoded by the U3 element of the LTR, shows better conservation (data not shown).

Based upon sequence homologies with HIV-1, the *tat* and *art* genes of HIV-2 are probably organized as split genes transcribed into ~2 kb mRNA made of three exons^{18,28–31}: the 5' leader, a first coding exon located in the central region and probably ending at a possible splice donor found at position 6,111 (CAAGT, Fig. 2), and a last exon probably starting at the splice acceptor at position 8,307 in HIV-2 (CAGATC). The *tat* protein of HIV-2 would be longer than that of HIV-1 (130 versus 86 amino acids), having two large insertions in the amino terminus and in the second coding exon (Fig. 4). The main domain of homology of the *tat* proteins corresponds to a region very rich in cysteine residues whose structure is reminiscent of that of the 'cysteine fingers' of some transcription-regulating elements that interact with nucleic acids, such as the TFIIIA factor⁴⁰. This region is followed by an Arg-Lys-rich stretch that could also interact with DNA or RNA. No significant homology is seen in the second coding exon, which has been shown to be dispensable to the function of the protein^{28,29}. The *art*-encoded protein is shorter in HIV-2 than it is in HIV-1 (100 versus 116 amino acids), and most of its length is encoded by the last exon. The most homologous part is located in a stretch of basic residues that may be able to interact with nucleic acids.

Cross-transactivation of HIV-1 and HIV-2

The trans-activator gene (*tat*) has been shown to be indispensable for the replication and cytopathicity of HIV-1 (ref. 41).

Table 1 Quantification of the homologies among retroviral proteins

a	HIV-1	GAG	POL	ENV		F	Central Region			
				EGP	TMP		Q	R	tat	art
HIV-2		57.7 (95.2)	59.4 (96.6)	39.4 (90.6)	44.8 (91.3)	37.7 (79.7)	34.6 (87.4)	52.2 (85.7)	42.8 (84.6)	44.8 (97.7)
b										
	HIV-2	HIV-1	HTLV-I	VISNA						
HIV-1	59.1 (96.4)	—	ND	ND						
LAV-Eli	61.6 (96.1)	94 (98.7)	ND	ND						
LAV-Mal	59 (95.2)	92 (98.7)	ND	ND						
EIAV	43.8 (92)	41.9 (91.5)	ND	46.7 (90.8)						
VISNA	43.7 (88.7)	42.2 (94)	ND	—						
HTLV-I	34.8 (70.5)	33.3 (70.3)	—	ND						
HTLV-II	ND	ND	62.8 (99.5)	ND						
BLV	ND	ND	49.5 (93.2)	ND						
RSV	35.9 (72.3)	34.5 (76.2)	38.2 (86.4)	ND						

The reference protein of each alignment is that listed at the top of the column. Proteins were aligned using the NUCALN program⁶¹ with following parameters: K-tuple 1, window 20, gap penalty 1. Two results are indicated in each case: the amino-acid identity (%) in the aligned domains (that is, excluding the regions of insertion/deletion), and between parentheses the percentage of the length of reference protein that could be aligned. a, Homologies between HIV-1 and HIV-2 proteins. For *env*, the calculation was done for the external glycoprotein (EGP, including the signal peptide, whose length is not exactly known in HIV-2), and the transmembrane protein (TMP). b, Comparison of the *pol*-encoded proteins of different retroviruses. LAV-Mal and LAV-Eli are Zairian isolates of HIV-1 (ref. 12); EIAV: equine infectious anaemia virus (sequence communicated by Dr S. Aaronson), and visna virus²⁷ are animal lentiviruses; HTLV-I, HTLV-II, BLV^{62–64}, related leukemogenic retroviruses; RSV Rous sarcoma virus⁶⁵. ND, not determined.

ENV		EGP	
HIV-2	1KXKLLIAALLA--SALVYCTOTVTVFVCTVTKKATILPLCATANE--DT-----GCTIQCLPDDDDYQET--LYTEAFDAQNTV	79
HIV-1	1KXKLLIAALLA--SALVYCTOTVTVFVCTVTKKATILPLCATANE--DT-----GCTIQCLPDDDDYQET--LYTEAFDAQNTV	100
signal		EGP	
HIV-2	2	TEGALIDVWMLFETSIRPEVCLTPLVAVLSTESSTGHMTTSKSTSTSTTTT--PTDQ--QDISEDTP--RADNCSGLGKETIRCOFNGGLERDKKQ	177
HIV-1	1	TEGALIDVWMLFETSIRPEVCLTPLVAVLSTESSTGHMTTSKSTSTSTTTT--PTDQ--QDISEDTP--RADNCSGLGKETIRCOFNGGLERDKKQ	187
HIV-2	2NET--WISKDVKVCTENNSMTQCTNNHNTSVITESCKHYVDAIRFRTCAPPGYALLRNDOT--NYSGFAPRCSRVVASTCTENHGTQSTSTV--GF	271
HIV-1	1NET--WISKDVKVCTENNSMTQCTNNHNTSVITESCKHYVDAIRFRTCAPPGYALLRNDOT--NYSGFAPRCSRVVASTCTENHGTQSTSTV--GF	266
HIV-2	2GCTRAEN--RTTSTVNGRDN--RTIL--SLNKTMLSLHCKRGRKTVQ--NLMH--GIVFNSHTQPIKAPROAMCWFEC--SKDANDVETLLEHPE	362
HIV-1	1GCTRAEN--RTTSTVNGRDN--RTIL--SLNKTMLSLHCKRGRKTVQ--NLMH--GIVFNSHTQPIKAPROAMCWFEC--SKDANDVETLLEHPE	356
HIV-2	2YRGNTDTRHISFAAPKGSQSPVAYMTNCECEFLCTNHTVFLN--VI-----ENKTHRYAPN--IKQIINTVNGVNVPLPREGLESLST	449
HIV-1	1YRGNTDTRHISFAAPKGSQSPVAYMTNCECEFLCTNHTVFLN--VI-----ENKTHRYAPN--IKQIINTVNGVNVPLPREGLESLST	453
EGP		TMP	
HIV-2	2VTSIIAMIDVWMLFETSIRPEVCLTPLVAVLSTESSTGHMTTSKSTSTSTTTT--PTDQ--QDISEDTP--RADNCSGLGKETIRCOFNGGLERDKKQ	542
HIV-1	1VTSIIAMIDVWMLFETSIRPEVCLTPLVAVLSTESSTGHMTTSKSTSTSTTTT--PTDQ--QDISEDTP--RADNCSGLGKETIRCOFNGGLERDKKQ	548
EGP		TMP	
HIV-2	2LLAGIYQDQQLLDVYKQQLLRLTVMGTKLQARVATALEKYLQDQARLNSWGLAPROVCHTVPV--VVD--LAPMNDHTVQVETLLEHPE	638
HIV-1	1LLAGIYQDQQLLDVYKQQLLRLTVMGTKLQARVATALEKYLQDQARLNSWGLAPROVCHTVPV--VVD--LAPMNDHTVQVETLLEHPE	648
HIV-2	2KSLAQIQQEKNNYELQQLNSWGLAPROVCHTVPV--VVD--LAPMNDHTVQVETLLEHPE	737
HIV-1	1KSLAQIQQEKNNYELQQLNSWGLAPROVCHTVPV--VVD--LAPMNDHTVQVETLLEHPE	740
HIV-2	2DQCSNGDRTVMPFATYINFLIRQLRLT--RLYSICDRLSRSFLTLQIYQNLQMLRLTA--FLQTGCMIQEAFQ--AAARATETL--	824
HIV-1	1DQCSNGDRTVMPFATYINFLIRQLRLT--RLYSICDRLSRSFLTLQIYQNLQMLRLTA--FLQTGCMIQEAFQ--AAARATETL--	833
HIV-2	2ACAGCLWVLERICGCLAVPRIRIGQRIALL	858
HIV-1	1ACAGCLWVLERICGCLAVPRIRIGQRIALL	861
Tat			
HIV-2	2METPLKAPRESSIKLSNEFFSRTSODVATQELARQCEELISQLRPLETCHNSCTCKACQYHQMCF/LNKGGLGCTYERKGR--RATFETETPSPPTD	98
HIV-1	1METPLKAPRESSIKLSNEFFSRTSODVATQELARQCEELISQLRPLETCHNSCTCKACQYHQMCF/LNKGGLGCTYERKGR--RATFETETPSPPTD	71
HIV-2	2ASTRTGDSQPTKQKATVETDTCGR	130
HIV-1	1ASTRTGDSQPTKQKATVETDTCGR	86
Art			
HIV-2	2NNER--AD--EGLQKRLRLIILLQNTYPOGCTASQRNRRRRRQRWQILALADS--TTF--PDPPADSP--DQTIQGLT--	81
HIV-1	1NNER--AD--EGLQKRLRLIILLQNTYPOGCTASQRNRRRRRQRWQILALADS--TTF--PDPPADSP--DQTIQGLT--	100
HIV-2	2QELPDPPTPLPESORLAET	100
HIV-1	1QELPDPPTPLPESORLAET	116

Fig. 4 Alignments of the HIV-1 (BRU isolate, ref. 15) and HIV-2 proteins. Asterisks indicate amino-acid identities. Gaps were introduced to optimize the alignments. In the envelopes, the potential cleavage sites are shown by arrows. EGP, external glycoprotein; TMP, transmembrane protein. \diamond , Potential N-glycosylation sites; \bullet , cysteines. The domains of the EGP of HIV-1 that were found to be well-conserved among isolates¹² are underlined. The parts of *lat* and *art* encoded by each of the two exons are separated by an arrow.

To examine whether transactivation (a property also shared with the ovine visna lentivirus but not with the related caprine arthritis and encephalitis virus⁴²) exists in HIV-2, we constructed a plasmid, called pHIV2-CAT, containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the U3-R region of HIV-2 (225 bp of U3 and 175 bp of R). To test the transactivation of HIV-2, cells were either infected with HIV-2 or mock-infected, and five days later transfected with either pSVCAT (which contains the CAT gene under control of the SV40 early promoter⁴³) or pHIV2-CAT. At the time of transfection, the cells were not producing virus. Nonetheless, we observed a substantial increase in the amount of CAT expression in extracts of HIV-2-infected versus mock-infected cells that had been transfected with pHIV2-CAT (Fig. 5a). The expression of the SV40 early promoter was not affected by HIV-2 infection.

To determine whether the *tat* gene of HIV-1 could transactivate the LTR of HIV-2 and vice versa, we cotransfected SW480 cells⁴⁴ with subgenomic fragments of HIV-1 or HIV-2 and pHIV2-CAT or a plasmid called pHIV1-CAT, which contains U3-R of HIV-1 (the entire U3 and 70 bp of R) directing transcription of the CAT gene. The plasmid pLET (a gift from Dr S. Wain-Hobson) contains the region of the HIV-1 shown by others to encode the HIV-1 *tat* gene^{28,29}. The plasmid pME214, on the other hand, contains HIV-2 sequences between nucleotides 5,786 and 8,571 (Fig. 2), and in particular contains the open reading frames of HIV-2 that share homology with the *tat* gene of HIV-1. In both of these plasmids transcription is driven

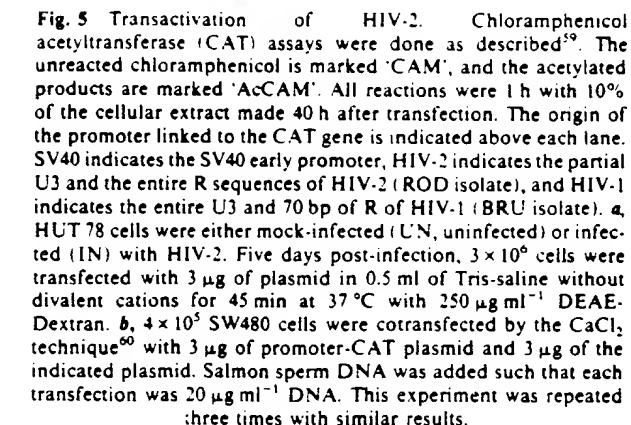
by the LTR of the respective virus, and the first AUG of the transcript is the first AUG of the putative *lat* gene. It should be noted that both these plasmids also contain the coding potential for the *art* gene.

Although the SV40 early promoter was not affected by either the HIV-1 *tat* nor the HIV-2 *tat* genes, both HIV-1 and HIV-2 LTRs were substantially activated by the HIV-1 *tat* gene (Fig. 5b). This is perhaps surprising in view of the difference in size of the R region of HIV-1 (where the transactivator responsive region (TAR) resides⁴⁵) and HIV-2. However 35 of the 58 bases present in the first stem-and-loop secondary structure of the TAR region of HIV-1 are conserved, and an analogous stem-and-loop structure with the first 77 bases of R can be drawn for HIV-2 (ref. 33).

The HIV-2 LTR is transactivated over 100-fold by pME214 (Fig. 5b). On the other hand, the HIV-1 LTR is not as well transactivated by this plasmid (~5-20 fold, Fig. 5 and other data not shown). Similar results were obtained after transfection of HeLa and HUT 78 cells (data not shown). These experiments indicate that pME214 encodes a functional *tat* gene. In addition, they indicate that the specificity of the HIV-2 *tat* is somewhat different from that of the HIV-1 *tat*. It will be important to determine whether this observation is isolate-specific.

Origin of human immunodeficiency viruses

We have presented here the complete nucleotide sequence of the retrovirus associated with AIDS in West Africa, HIV-2, and tentatively identified the viral proteins either detected in



The almost simultaneous emergence of two foci of AIDS in distinct areas of the African continent is unlikely to be due to the recent emergence of two novel human pathogens, for example by simultaneous trans-species infection by animal retrovirus, or by the mutation of pre-existing non-pathogenic human retroviruses. Indeed, HIV-1 and HIV-2 are obviously retroviruses with a common origin, but they are highly divergent, and it is more likely that their time of divergence is earlier than the beginning of the current epidemics. Therefore a common ancestor, with similar properties and pathogenic potential, prob-

A question to be addressed is why the HIVs were only recently detected if they existed for a long period. This may be due to the fact that the pathogenicity of an HIV-type retrovirus cannot be revealed until it has spread to a significant portion of the population. First, in areas of Africa with poor medical facilities where other infections, such as malaria, represent primary causes of morbidity, isolated cases of AIDS could have been an undetectable clinical event. Then, the incubation time can vary considerably, and it cannot still be ruled out that a large fraction of individuals infected by a HIV will remain healthy carriers. In Kenya, HIV-1 seropositivity was first reported in a high fraction of subjects at risk of AIDS (female prostitutes) who were apparently healthy; later, the virus diffused to a larger portion of the population, and cases of AIDS were observed⁴¹. A similar situation could explain the apparent lack of pathogenicity of the retrovirus designated HTLV-IV, but indistinguishable from HIV-2 and SIV by the antigenicity of its proteins^{19,48,49}. The presence of HTLV-IV was identified only in apparently healthy individuals in West Africa, an area where we have observed several typical AIDS cases caused by HIV-2. It is possible that the apparent non-pathogenicity of HTLV-4 is due to a recent epidemic diffusion of HIV-2/HTLV-IV in the West Africa, where AIDS cases still represent a minor fraction of the infected and seropositive individuals, whereas HIV-1 has diffused in majorities of central Africa or the USA some time before.

HIV-1 and probably HIV-2 recognize the CD4 surface molecule as a receptor on helper/inducer T lymphocytes and perhaps on other cells expressing the CD4 protein⁵⁰⁻⁵³. In HIV-1 this interaction is mediated by the external envelope glycoprotein (EGP; ref. 52), and an important problem is which of the domain(s) of this protein are involved in that interaction. Indeed, blocking this step of the virus life cycle, either by antibodies or drugs, could be an efficient means for preventing infection or blocking its spread. As the receptor is a constant cellular protein, we can postulate that the binding domain of the envelope is conserved among the CD4-tropic HIVs. The conserved domains of the EGP of HIV-1 and 2 are not numerous and therefore it becomes possible to demonstrate their possible role in the virus-receptor interaction using a relatively limited

set of site-directed mutations. Given the absence of antigenic cross-reactivity of the envelopes of the two HIVs, this CD4-binding domain is probably not, or only poorly, immunogenic—perhaps because of masking by glycosylation, poor exposure on the virion surface, or mimicking of 'self' antigens. Nevertheless, its presentation to the immune system out of context of the virion, that is, as a peptide, might induce a neutralizing antibody response that is not attained, or attained with only a low efficiency, with the complete native envelope from virions or expression systems⁵⁴⁻⁵⁶.

Conclusion

The comparative analysis of HIV-1 and 2 reveals major genetic differences between retroviruses that share many of their biological properties. They both cause AIDS, are cytopathic *in vitro*,

have a tropism for CD4-bearing cells and have elements *trans*-activating the expression of viral genes acting at the LTR level. The evolutionary potential of these viruses is therefore striking, and we must ask whether other HIVs can emerge as long as a favourable epidemiological situation is provided. We must take advantage of the precise delineation of the conserved structures to understand their molecular biology and develop new therapeutic tools, especially immunoprophylactics.

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LETTERS TO NATURE

Switching phenomena in a new 90-K superconductor

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Recently, Wu *et al.*¹ and Hor *et al.*² have shown that $Y_{1.2}Ba_{0.8}CuO_{4-x}$ is a superconductor with a superconducting onset temperature at ~ 92 K as determined by their resistivity and a.c. susceptibility measurements. Because the magnetic properties are important in describing the nature of superconductivity, we have measured the d.c. magnetic moment of this material. Here we show that this material cooled in zero field or in a high field ($H_{cool} >$

90 G) is diamagnetic below $T_{cm} \approx 90$ K, consistent with the previous measurements^{1,2}. However, when the sample is cooled in a small field (≤ 85 G), the magnetization, M , first becomes negative (diamagnetic) below T_{cm} , but further cooling results in a jump of M to a positive value at low temperature. We have also observed this switching by the application of an additional small field when the sample was cooled in a small field.

The $Y_{1.2}Ba_{0.8}CuO_{4-x}$ sample was prepared as described in ref. 1. The X-ray diffractograms reveal that the sample has multiple phases, devoid of the K_2NiF_4 structure. From the electrical resistance measurement, the superconducting onset temperature is $T_{\infty} \approx 94.5$ K and the resistance becomes 'zero' below $T_0 = 92$ K indicating that the sample is a superconductor with a rather narrow transition width. A Quantum Design superconducting quantum interference device (SQUID) magnetometer has been employed to measure the magnetization of the sample as a function of temperature and magnetic field. When the sample is cooled under zero field conditions, we have found that M is diamagnetic below T_{cm} and the susceptibility below ~ 25 K reaches $\sim 35\%$ of that of perfect diamagnetism ($-1/4\pi$).

We have also measured M when the sample is cooled in a field, H_{cool} . In Fig. 1, the magnetization obtained at various